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14. ABSTRACT

Adenovirus (Ad)-mediated transduction of dendritic cells (DCs) is inefficient because of the lack of the primary Ad receptor, CAR. CD40 is a surface marker expressed by DCs that plays a crucial role in their maturation and subsequent stimulation of T cells. DC infection with Ad targeted to the CD40 results in increased gene transfer. Cells transduced with CD40-targeted Ad5-SV40-TAg vector showed increased expression of transgene and expression of co-stimulatory molecules at 48 hours post-infection compared to cells transduced with untargeted Ad5-SV40-TAg vector. We demonstrated that CD40-targeted gene transfer promotes DC maturation with induction of a complex signaling cascade accompanied by characteristic changes in cytokine production. These results demonstrate that DCs can be successfully transduced using a CD40 targeted adenoviral vector and that transduced DCs show activation.

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BODY

1. BACKGROUND

1.1 Immunotherapy Strategies for Cancer Treatment: The combined effort of many researchers during the last thirty years has provided significant progress understanding the immunological features of cancer cells. Most cancers possess tumor-specific antigens, or overexpress antigens present in normal tissues, that can serve as targets of the immune system [1]. Despite this, it is obvious that upon the onset of cancer, the immune system fails to effectively mount a cellular anti-tumor response able to promote tumor rejection. The bases for this failure have just begun to be elucidated. Immunological ignorance of tumor antigens is due to an imbalance in the combination of signals between cancer cells and T cells, necessary to initiate an immune response [2]. In particular, interaction between MHC class I molecules in the tumor cells and the T cell receptor (signal 1) and between adhesion/costimulatory molecules (signal 2) are both necessary for cytotoxic T lymphocyte (CTL) activation. In accordance with this model, tumor cells fail to activate T cells because errors at one or both signals occur. Down regulation of MHC class I molecule expression (signal 1), and lack of co-stimulatory molecules (signal 2) are defects that render tumor cells invisible to the immune system. Re-establishment of these signals by delivery of cytokines, costimulatory molecules, and even MHC antigens have all been approaches used for cancer immunotherapy (reviewed in [3,4]). The growing understanding of the role of cytokines in the regulation of anti-tumor responses led to clinical trials administering recombinant cytokines such as IL-2, IFN-γ, and IL-12 systemically, with only limited success due to high toxicity [ref]. Another approach involved the transfer of cytokine genes (*e.g.*, IL-2, IL-4, IL-7, IL-12, GM-CSF, or IFN-γ) in autologous/allogeneic fibroblasts or tumor cells cultured and irradiated *ex vivo* followed by reinfusion into the patient [ref]. This approach requires isolation of tumor cells from patients from which to establish primary tumor cell lines for transduction. In order to circumvent this limitation and to restrict cytokine delivery to cancer tissue, direct gene transfer systems have been used to achieve *in vivo* administration of cytokines [ref]. Both viral and nonviral methods have been used. Because of their high capacity and robust gene expression, recombinant Ads have received much attention as delivery vehicles for several interleukin genes, including IL-12, GM-CSF, and IL-2 [ref]. Despite the elicitation of initial anti-tumor responses in animal models, the inherent immunigenicity of the adenoviral vector prevents secondary administration, which may be necessary for long-lasting immunity. The identification of tumor associated-antigens recognized by T cells has opened new directions for immunogene therapy. DNA encoding tumor antigens has been used to immunize patients. Naked DNA vaccination approaches, in which a plasmid encoding a tumor antigen is administered subcutaneously or intradermally have proven useful in eliciting antibody and cellular anti-tumor responses. Recombinant virus-mediated delivery of tumor antigens into the skin or muscle have also proven to elicit anti-tumor responses. Currently, more effective methods to induce immune responses against tumor antigens are being developed based on the use of professional antigen-presenting cells (APC). The presentation of tumor antigens released upon tumor cell death and captured by APC (a phenomenon of "cross- presentation') has been proposed as a more potent strategy than direct presentation of tumor antigens by the tumor cells themselves.

1.2. The Dendritic Cells as a Candidate Effector Cell for Presentation of Tumor Antigens: Dendritic cells (DCs) are the most potent APCs so far identified and as such, they are also the most potent initiators of immunity [9,10]. They can capture, process and present antigens in combination with MHC class I and class II molecules to naive CD8+ (cytotoxic) and CD4+ (helper) T lymphocytes, respectively. Through this process, specific cytotoxic T lymphocytes for that antigen are activated that can recognize a target cell and kill it. DCs possess particular characteristics that make them suitable for antigen presentation. They are found in different maturation cell states that confer them distinct abilities. In their

immature state, DCs are predominantly antigen-capturing cells, through phagocytosis, macropinocytosis and receptor-mediated endocytosis. Upon capturing, antigens enter the endocytic pathway where they are processed and loaded in MHC class II molecules for presentation to CD4-expressing T lymphocytes. Antigens that have access to the cytosol, like viral proteins synthesized inside the DC upon infection, can associate with MHC class I proteins and be presented to CD8+ T cells. The exogenous pathway, in which antigens not synthesized inside the DC can also be presented in association with MHC class I, is a mechanism recently identified and is responsible for cross-presentation. In this regard, it has recently been shown that DCs can acquire antigens from apoptotic cells through a receptor-mediated mechanism that can generate class I-restricted responses [11]. This would explain how DCs can process and present, in an MHC class I-restricted manner, antigens from transplanted or tumor cells, that otherwise would not be cytosol-accessible. Immature DCs are found at many tissues, in particular at the port of entry of infectious agents such as skin (the Langerhans cells). Although immature cells are specialized in capturing and processing, their T-cell activation ability is very limited. DC maturation can be driven by antigens themselves, by cytokines, and by interaction with T cells. In the mature state, DCs up regulate their expression of MHC , and costimulatory proteins necessary for T cell activation. They also acquire their distinctive stellate shape and increase their motility allowing them to migrate to lymphoid centers where antigen presentation takes place. Specific naive cytotoxic and helper T cells are thus activated and the immune response initiated. It is upon the interaction with T cells that DCs acquire their final maturation phenotype; characterized by loss of phagocytic capacity, further expression of costimulatory molecules, and synthesis of cytokines such as IL-12. This final maturation step is not insignificant. It has recently been described that interaction of DCs through CD40 with T helper cells expressing CD40 ligand (CD40L) can bring the DC to a state that triggers cytotoxic T cell responses [12-14]. Therefore, this final maturation based upon CD40-CD40L interactions allows DC to present antigens and activate CD8+ T cells. Shortly after complete maturation, DCs undergo apoptosis and die.

1.3 The Use of Dendritic Cells in Tumor Vaccination Approaches: The use of DCs as adjuvants for cancer therapy was boosted by the development of techniques to generate and culture sufficient amounts of DCs *ex vivo*. DCs originate from a hematopoietic precursor and they represent a low percentage in the circulation. Investigators have been able to derive DCs from different sources such as peripheral blood monocytes or bone marrow CD34+ cells, using the appropriate combination of cytokines. It is now feasible to obtain enough number of DCs and stimulate them *ex vivo* to present tumor-specific antigens. Subsequently, autologous DCs can be reinfused into the patient by intravenous or intradermal administration, and cells travel to the lymph node where presentation and activation of T cells take place. A number of reports have shown preclinical data on the potency of DC-based vaccination [15-35], and phase I and II clinical trials targeting different types of tumors have been initiated [36-38]. The choice of current immunogen and vaccination protocols is broad: the immunogens typically used in DC-based vaccination fall into two major categories: single tumor antigens and multiple antigen mixtures. The rationale of using single defined tumor antigens is that some tumor antigens and even peptide fragments interacting with different MHC class I alleles have been identified and isolated. From a pharmacological standpoint, this approach provides a cleaner immunization system. However, one drawback is that negative selection of antigen-deficient cells may also occur. Immunization with mixtures containing multiple tumor antigens represents an alternative approach. In this case, unfractionated tumor material is generally used and therefore may contain proteins common in normal tissues. Although this approach may increase the chances of presentation of weak or undefined tumor antigens, it may also give rise to autoimmune responses. The most widespread approaches in vaccination protocols have been to load (pulse) dendritic cells with synthetic peptides that correspond to tumor-associated antigens recognized by CD8+ T cells in combination with a defined MHC allele. Using this

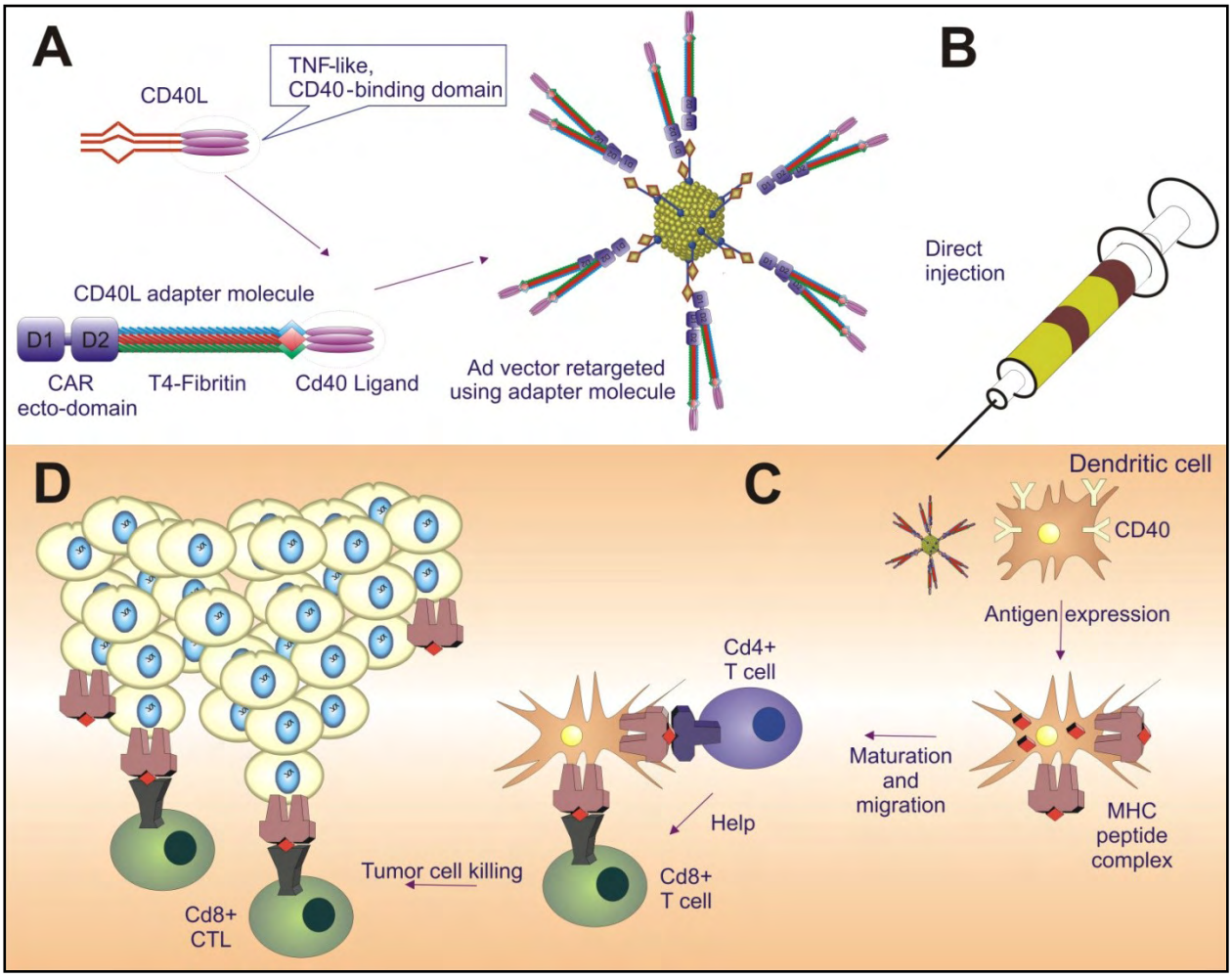
protocol, cells are co-cultured with the peptide, which in turn, associates with class II and also binds to class I molecules. This method has been shown to promote protective anti-tumor immunity and regression of pre-established tumors [17,20,22,32,39,40]. The main disadvantage of this approach is that not all tumor antigens are known for many tumors and not all MHC class I-restricted peptides are known for a given tumor-specific antigen. For this reason, DCs have been pulsed with peptides eluted from MHC-antigen complexes present in tumor cells [16]. This approach has shown therapeutic effects in weakly immunogenic tumors in experimental models. In the same regard, DCs have been pulsed with total soluble antigens from tumor extracts and shown to elicit tumor-specific immunity [28,41,42]. Although both eluted tumor peptides and tumor lysates do represent individualized approaches for each patient's tumor, the fact that they rely on the availability of tumor material restricts their use. Different approaches based on genetic transduction, whereby a coding sequence for a tumor antigen is introduced and expressed in DCs, have also been attempted. In this case, most of the endogenously synthesized antigen is channeled mainly into class I molecules and CD8+ responses are elicited. By providing the complete DNA sequence for a tumor antigen, presentation is not limited to a single epitope for a given MHC allele. Furthermore, the delivery of genetic material allows for prolonged expression and therefore presentation of antigens. Both nonviral [21,26,43-45] and viral [22,25,28,33,35,42,46-53] methods have been described for introduction of DNA sequences, which have shown to promote specific CTL responses and protective and therapeutic anti-tumor immunity of tumors expressing the antigen sequence delivered. However, because the endogenously expressed antigens may have only limited access to the class II presentation pathway, it is possible that there will be a deficiency in generating CD4+ T helper cells, required for an effective and durable CTL response. An approach different from *ex vivo* DC pulsing and genetic transduction is the use of cell fusion between tumor cells and DCs [30,54,55]. This approach combines the presence of tumor antigens in tumor cells with the presentation capability of DCs. While the advantage of this approach may be the presentation of undefined tumor antigens both to CD4+ and CD8+ T lymphocytes, there is still some concern regarding the generation of autoimmune responses. In addition, the requirement of viable autologous tumor cells is a disadvantage for clinical implementation.

1.4 Dendritic Cells as Targets for *In Vivo* Vaccination: The promise of DC-based vaccination strategies is reflected in the fact that more than 50 DC-related clinical trial protocols have been published (results reviewed in [56]). However, there are critical issues in the DC-based vaccination approaches that may limit their employment in the clinic. With the advent of DC culturing techniques *ex vivo*, researchers have sought DC-specific markers. Although a panel of characteristic markers has been defined (*e.g.*, CD1a+, CD40+, CD80+, CD86+, MHC class I and class II, ICAM-l, LFA-3), it is now obvious that the collective name of DC, groups a heterogeneous population of cells, derived from different lineages, in different maturation states, and probably showing distinct functional features [57]. Thus, differentiation of DCs *ex vivo* may be subjected to different environmental modifications resulting in cells that do not totally represent their counterparts *in vivo*. This may translate into reduced potency to elicit effective anti-tumor responses. Therefore, the development of an *in vivo* approach based on DC vaccination without involving isolation and culturing of DCs *ex vivo* would be clinically significant. However, a critical component of *in vivo* transduction is efficient targeting of the vector to DCs without perturbation of DC function. To this end, we have generated Ad vector systems that specifically targets human [59] and mouse [60] DCs via the CD40 receptor using CAR-CD40L bi-specific adapter molecules and have shown that CD40-targeted Ads efficiently transduce DCs *in vitro* without interfering with DC function. The availability of a DC-targeted Ad system makes possible the *next* step: namely, testing an anti-tumor vaccination model system *in vivo***.**

1.5 Summary: Dendritic cells (DCs) capture, process and present antigens in association with MHC class I and class II molecules to naive CD8+ cytotoxic and CD4+ helper T cells. Through this, specific cytotoxic T cells are activated, and recognize a target cell and kill it. This study was to determine the transduction efficiency of DCs using a CD40-targeted adenoviral vector expressing a tumor antigen. Recently, we characterized a new model using a mouse ovarian carcinoma cell line (IG10) that we have engineered to express the SV40 large T Antigen and forms tumors in syngeneic mice. The SV40 large T-Ag is highly immunogenic, inducing both antibody and cytotoxic T lymphocyte (CTL) responses. Since this antigen is synthesized in IG10 cell clones, the SV40 large T-Ag is an attractive candidate as a model system for the development of a DC-targeted cancer vaccine. We hypothesize that transduction of DCs in vitro using a CD40-targeted Ad5 vector expressing SV40 TAg (Ad5-SV40 TAg) will result in a high level of transgene expression, and be effective in inducing an antigen-specific CTL response. To target Ad5-SV40 Ag to DCs, we utilized a recombinant adapter protein consisting of extracellular portion of the native adenovirus receptor, CAR, fused to a trimerization motif from T4 fibritin protein, and linked to the extracellular domain of the mouse CD40 ligand (Figure 1).

Dendritic cells (DCs) are professional antigen presenting cells (APCs) that direct the cellular immune response through antigen presentation in the presence of appropriate co-stimulation [1]. Multiple techniques of antigen priming have been advocated to create a tumor-specific DC vaccine [2]. The expression of whole tumor antigens within the host antigen presenting cells following vaccination, has been demonstrated to result in the presentation of multiple tumor-associated epitopes in the context of MHC class I and/or class II molecules [2]. In the following pilot project, we propose to evaluate a dendritic cell-targeted adenovirus (Ad) vaccine expressing the simian virus 40 (SV40) large T antigen (T-Ag) in a mouse model of ovarian cancer. We hypothesize that immunization of dendritic cells with the SV40 T-Ag will be effective in inducing antigen-specific cytotoxic T-lymphocyte (CTL) responses, and suppress the growth of ovarian tumor cells expressing the SV40 T-Ag. In the following experiments, our goal is to utilize a model of ovarian cancer in mice with an intact immune system. Although the immunodeficient mouse is an important animal model for the preclinical development of new cancer therapies, the major disadvantage of this model is the absence of T cells that may modulate therapeutic effects. The use of mouse ovarian cancer cell lines to form tumors in normal immune-intact mice will provide a model in which immune interactions in the establishment, progression, and treatment of ovarian cancer can be investigated. In addition, the use of an immune-intact mouse model will provide a more realistic representation of human cancer and potential efficacy of gene manipulation strategies. There are only a few syngeneic models available which are derived from intraperitoneal ovarian tumors in mice. Recently, we have characterized a new model using a mouse ovarian carcinoma cell line (MOVCAR) that expresses the SV40 T-Ag and forms tumors in syngeneic immunocompetent B6C3F1 mice [4]. The SV40 T-Ag is highly immunogenic, inducing both antibody and cytotoxic T lymphocyte (CTL) responses [3]. Since this antigen is synthesized in MOVCAR cells, the SV40 T-Ag is an attractive candidate as a model system for the development of a DC-targeted cancer vaccine Therefore we have all of the reagents necessary to directly evaluate the efficacy of an *in vivo* dendritic cell-targeted Ad vaccine, using a syngeneic mouse model of ovarian cancer.

Figure 1.1
Targeting of Ad5 vector to CD40 with TNF-Like domain of CD40L provides flexible platform for DC infection



The development of an *in vivo* approach based on DC vaccination without isolation and culturing of DCs *ex vivo* would be clinically significant. However, a critical component of *in vivo* infection is efficient targeting of the vector to DCs without perturbation of DC function. To this end, we generated Ad vector systems that specifically target human and mouse DCs via the CD40 receptor using CAR-CD40L bi-specific adapter molecules (A) and showed that CD40-targeted Ads efficiently infect DCs *in vitro* without interfering with DC function. We evaluated a dendritic cell-targeted Ad vaccine expressing the simian virus 40 (SV40) large T antigen (T-Ag) in a mouse model of ovarian cancer. We hypothesize that immunization of DCs (B) with the SV40 T-Ag will be effective in inducing antigen-specific cytotoxic T-lymphocyte (CTL) responses (C), and suppress the growth of ovarian tumor cells expressing the SV40 T-Ag (D).

2 *IN SITU* ADMINISTRATION OF A CD40-TARGETED AD5-SV40

2.1 Introduction

The *in vitro* results showed that dendritic cells could be efficiently infected by CD40-targeted adenovirus. Infection by CD40-targeted adenovirus induced DC maturation as well as secretion of inflammatory cytokines and chemokines by DCs. The next goal was to determine the potency of the CD40-targeted adenoviral vaccine *in vivo*, *i.e.,* to determine if CD40-targeted adenovirus expressing the tumor antigen can infect dendritic cells *in vivo* and induce a tumor antigen-specific immune response. The gold standard for determining the efficacy of an anti-tumor vaccine in animal models is the disease free or increased survival of animals on challenge with tumor cells and/ or improved survival of tumor bearing animals. However, before moving to the survival studies, it is important to measure the immune response generated in the animals upon vaccination, because even though the immune response may have been effectively induced, the tumor cells may adopt mechanisms to evade detection and killing by the activated immune effector cells, thereby making it difficult to cause an improvement in the survival of the immunized animals*[9]*. The induced immune response can be quantified or monitored by several methods*[10]*.

The lytic activity of activated CD8 positive T-cells can be measured by the cytotoxicity assays. *In vivo*, it is the killing activity of the CD8 positive Cytotoxic T-cells that leads to the clearance of the tumor and survival of the animal. Thus, to measure the lytic activity of the CTLs is a relevant assay to measure the anti-tumor immune response. The *in vitro* CTL assay involves mixing of the splenocytes or purified CD8 positive T-cells from the immunized animals and the target tumor cells that have been labeled with chromium (^{51}Cr) *in vitro*. This assay requires the stimulation and expansion of the T-cells *in vitro*. In addition, the target cells are killed by the CTLs *in vitro[11]*. However, the *in vivo* cytotoxicity assay measures the lysis of the target cells that are killed by the CTLs *in vivo[12]*.

Both, the *in vitro* and the *in vivo* CTL assays were used to measure the cytolytic activity of the CTLs, induced by the CD40-targeted adenoviral vaccine expressing the target tumor antigen in the immunized mice. The B6C3F1 mice were chosen as the model system to evaluate the induced CTL responses. B6C3F1 mice are immunocompetent mice. The use of mice with an intact immune system is critical for understanding progression and treatment of the disease and to evaluate new therapeutic approaches. SV40 T-Ag was the chosen model tumor antigen because it is highly immunogenic, inducing both antibody and cytotoxic T lymphocyte (CTL) responses*[13]* . The target cell line chosen for the *in vitro* CTL assay is MOVCAR-2 because it is a mouse ovarian cancer cell line derived from the B6C3F1 mice that expresses the SV40 T Ag*[14]*. To test the validity of the use of MOVCAR-2 cells as target cells for cytotoxicity assay, expression of the target SV40 T-Ag in the MOVCAR-2 parental cell line and retention of this expression by MOVCAR-2 cells in tumors *in vivo* was confirmed by Western blot analysis. The expression of other components of the antigen processing and presentation pathway, such as MHC class I and **T**ransporter of **A**ntigenic **P**eptides-1 (TAP-1) was also confirmed in the MOVCAR-2 cells.

2.2 Materials and Methods

***In vitro* Cytotoxic T Lymphocyte assay: study of the cell line, MOVCAR-2, to be used as target in the *in vitro* CTL assay.**

Assay of Tumorigenicity. B6C3F1 mice were injected with MOVCAR-2 cells. 1 X 10^8 MOVCAR-2 cells were injected intraperitoneally into each B6C3F1 mouse. The mice were then monitored for tumor formation.

Analysis of SV40 T-Ag expression in MOVCAR-2 tumor cells by Western blot. The MOVCAR-2 cell line and tumor cells cultured from the ascites and solid tumor collected from B6C3F1 mice injected with the MOVCAR-2 cell line were analyzed for expression of the tumor antigen, SV40 T-Ag. The cells were harvested and lysates were prepared. Protein concentrations of each sample were determined using the Bradford method, and all lysates were normalized to the lowest concentration of protein. Samples were loaded onto 10% SDS-polyacrylamide gels, separated by electrophoresis, and transferred to nitrocellulose membranes (15 V for 15 min). Membranes were blocked for 1 h with 5% bovine serum albumin in Tris-Cl buffered saline (TBS), followed by two 5 min washes with 1% Tween-20 in TBS (TTBS). The membranes were incubated for 1 h in TTBS containing a primary mouse anti-mouse SV40 T-Ag antibody (BD Biosciences, clone PAb101), and washed three times for 30 min each with TTBS. Afterwards, the membranes were incubated for 1 h in TTBS containing a horseradish peroxidase-labeled goat anti-mouse IgG antibody, and washed three times for 30 min each with TTBS. Finally the membranes were developed using the ECL substrate (Amersham), and exposed to x-ray film.

Analysis of expression of MHC class I in MOVAR-2 cell line and MOVCAR-2 tumor cells by flow cytometry. MOVCAR-2 cell line and tumor cells cultured from the ascites and solid tumor collected from B6C3F1 mice injected with the MOVCAR-2 cell line were analyzed for expression of MHC class I. The MOVCAR-2 cells activated by addition of recombinant mouse IFN-gamma (R&D Systems) were used as the positive control. After 48 h of incubation *in vitro*, the cells were harvested and stained, and 5 X 10^5 cells were stained per well for MHC class I, *i.e.,* the H-2K^b (BD Biosciences clone AF6-88.5) and H-2K^k(BD Biosciences clone 36-7-5) haplotypes of MHC class I. The antibodies were used at 1 μg/ml. Labeled antibody were added to the cells of interest and incubated in the dark for 20 min at 4^0C. Isotype control antibodies were also used to set up compensation. The cells were fixed in 1% paraformaldehyde for 5 min in the dark, washed twice and resuspended in 200 μl of FACS buffer. The cells were analyzed using a FACScan flow cytometer (Becton Dickinson).

Analysis of expression of TAP 1 in MOVAR-2 cell line and MOVCAR-2 tumor cells by Western blot. MOVCAR-2 cell line and tumor cells cultured from the ascites and solid tumor collected from B6C3F1 mice injected with the MOVCAR-2 cell line were analyzed for expression of TAP 1. The MOVCAR-2 cells activated by addition of recombinant mouse IFN-gamma (R&D Systems) were used as the positive control. After 48 h of incubation *in vitro*, the cells were harvested and lysates were prepared. Protein concentrations of each sample were determined using the Bradford method, and all lysates were normalized to the lowest concentration of protein. Samples were loaded onto 10% SDS-polyacrylamide gels, separated by electrophoresis, and transferred to nitrocellulose membranes (15V for 15 min). Membranes were blocked for 1 h with 5% bovine serum albumin in Tris-Cl buffered saline (TBS), followed by two 5 min washes with 1% Tween-20 in TBS (TTBS). The membranes were incubated for 1 h in TTBS containing a primary mouse anti-mouse TAP 1 (Santa Cruz Biotechnology catalog # 11465), and washed three times for 30 min each with TTBS. Afterwards, the membranes were incubated for 1 h in TTBS containing a horseradish peroxidase-labeled goat anti-mouse IgG antibody, and washed three times for 30 min each with TTBS. Finally the membranes were developed using the ECL substrate (Amersham), and exposed to x-ray film.

Generation Of Cytotoxic T-Lymphocytes (CTLs). CTLs were generated by immunizing B6C3F1 mice with Ad5-CMV-SV40 T-Ag complexed with CFm40L and Ad5-CMV-SV40 T-Ag alone. Each mouse was injected with 10^9 i.f.u. of Ad5-CMV-SV40 T-Ag alone and 10^9 i.f.u. of Ad5-CMV-SV40 T-Ag complexed with 1200 ng of CFm40L. A second dose was administered two weeks later. At ten days after the second immunization, the mice were sacrificed and spleens were collected. Splenocytes containing the CTLs were co-cultured with a synthetic peptide (CKGVNKEYL) that corresponds to an epitope of SV40 T-Ag for 5 days.

Chromium Release Assay. The cytotoxicity of the generated CTLs was determined in a standard 4-h chromium release assay. The target MOVCAR-2 cells (Interferon-gamma treated as well as untreated) and CMT.64 cell line (negative control) were labeled with 100 □Ci of $Na_2{}^{51}CrO4$ for 1.5 h. 1 X 10^3 labeled target cells were then co-cultured with different numbers of effector T-cells in a 96-well (v-bottom plates) for 4 h. At the end of the culture, 100 □ 1of supernatants were collected and radioactivity was measured on a gamma-counter. Maximum and spontaneous release of ^{51} Cr was obtained from the supernatants of the target cells in 1% Nonidet P-40 and in medium alone respectively. All experiments were set up in triplicate wells. Percent Specific lysis was calculated by the following formula*[11]*:

% specific lysis = (Experimental c.p.m. - Spontaneous c.p.m.) / □(Maximal c.p.m. - Spontaneous c.p.m.)□ □100.

***In vivo* Cytotoxic T Lymphocyte assay.**

Generation of Cytotoxic T-Lymphocytes (CTLs). CTLs were generated by immunizing B6C3F1 mice with different doses of Ad5-CMV-SV40 T-Ag and Ad5-CMV-SV40 T-Ag complexed with CFm40L. Different doses of Ad5-CMV-SV40 T-Ag used for the immunization were as follows: 10^9, 10^8, 10^7, 10^6, 10^5, 10^4, 10^3 and 10^2. 10^9 i.f.u. of Ad5-CMV-SV40 T-Ag was complexed with 1200 ng of CFm40L, 10^8 i.f.u. with 120 ng, 10^7 i.f.u. with 12 ng, 10^6 i.f.u. with 1.2ng, 10^5 i.f.u. with 0.12 ng, 10^4 i.f.u. with 0.012 ng, 10^3 i.f.u. with 0.0012 ng and 10^2 i.f.u. with 0.00012 ng of CFm40L. Mice that only received saline were used as the negative control. The mice received the same second dose of immunization, with the same virus, two weeks later. At 10 days after the second immunization, the mice were used for the *in vivo* CTL assay.

***In vivo* CTL assay**. Target cells for the *in vivo* CTL assay were prepared by making splenocytes from the spleens of naïve B6C3F1 mice. The erythrocytes were lysed from the splenocytes by using the ACK lysis buffer. The spleen cells were then divided into two populations. One population was pulsed with peptides (CKGVNKEYL and VVYDFLKC) corresponding to the antigenic epitopes of SV40 T-Ag by co-incubating the cells and each peptide (2.5 μg/ml in the culture medium) at 37^0C for 90 min. The second control population was not pulsed with any peptide. The pulsed splenocytes were labeled with a high concentration of CFSE (5 μM) and the non-pulsed splenocytes were labeled with a low concentration of CFSE (0.5 μM). The pulsed and the non-pulsed splenocytes were then mixed 1:1 and injected intravenously into B6C3F1 mice that were already immunized using Ad5-CMV-SV40 T-Ag. Mice that received only saline were used as the control mice. 2 X 10^7 cells were injected in each mouse in 500 μl of PBS. After 5 h., the mice were sacrificed and their spleens were harvested. The spleen cells were analyzed by flow cytometry and the two cell populations were detected by their different CFSE fluorescence intensities. The percentage of specific cell lysis was calculated using the following formulas*[12]*:

Ratio = (percentage of $CFSE^{low}$/ percentage of $CFSE^{high}$)

Percentage specific lysis = [1- (ratio unprimed/ratio primed) X 100]

2.3 Results

Analysis of SV40 T-Ag expression in MOVCAR-2. MOVCAR-2 is a mouse ovarian carcinoma cell line that expresses SV40 T-Ag and forms tumors in syngeneic immunocompetent B6C3F1 mice. Prior to using MOVCAR-2 cells as target cells to measure the induced cytotoxic T-cell response against SV40 T-Ag, it was important to confirm the expression of SV40 T-Ag in the parental cell line and in tumors formed *in vivo*. Thus, to determine expression of SV40 T-Ag, in MOVCAR-2 tumors, 1 X 10^8 cells were injected i.p. into B6C3F1 mice. The mice were monitored for tumor growth; after approximately 8 weeks, the mice started to show signs of tumor growth and were sacrificed and examined histopathologically. The MOVCAR-2 cells formed both ascites as well as solid tumors *in vivo*. The cells from the ascites as well as solid tumors were cultured *in vitro* and tested for the expression of SV40 T-Ag. As shown in Figure 2.1, the tumor cells cultured from both, the solid tumors (lane 1) and ascites (lane 2) showed expression of SV40 T-Ag. The parental MOVCAR-2 cell line (lane 3) expresses SV40 T-Ag and was used as the positive control. Thus, MOVCAR-2 cells from parental cell line as well as *in vivo* tumors demonstrate expression of SV40 T-Ag as shown by Western blot analysis.

Analysis of cell-surface expression of MHC Class I in MOVCAR-2. Along with expression of target tumor antigen, it was also important to determine expression of cell surface MHC class I and other components of the antigen processing and presentation pathway. Down-modulation of these molecules is one of the methods adopted by tumor cells to evade the immune system. Target tumor antigens can only be recognized by the activated lymphocytes if they are presented on the cell surface of the tumor cells along with MHC molecules. The appropriate cell surface presentation of the tumor antigen requires processing and presentation of the antigen along with MHC by the cellular antigen processing and presentation pathway. Expression of MHC as well as other components of the antigen processing pathway can be induced in the cells by exposure to IFN-gamma. Expression of MHC class I on MOVCAR-2 cells, from parental cell line as well as cells cultured from ascites and solid tumors was determined by flow cytometry. These cells were also treated *in vitro* with IFN-gamma to measure the induction of MHC class I after exposure to IFN-gamma.

At 48 h after treatment with IFN-gamma, the cells were harvested and analyzed for induction of MHC class I expression on the cell surface. As shown in Fig. 2.2, only 10% of the cells from the parental MOVCAR-2 cell line were positive for the K^b-haplotype of MHC class I. However, the expression of this haplotype increased to 89% when the cells were treated with IFN-gamma. In addition, only 12% of the cells were positive for the K^k-haplotype of MHC class I. The expression of K^k-haplotype, similar to of K^b-haplotype, increased to 87% when the cells were treated with IFN-gamma. As shown in Fig. 2.3, there was no detectable expression of both, the K^b as well as K^k haplotype on MOVCAR-2 cells cultured from the ascites. However, the expression of the K^b-haplotype increased to 79% of the cells and that of K^b-haplotype increased to 84% of cells after exposure to IFN-gamma. The MOVCAR-2 cells cultured from the solid tumors, as shown in Fig. 2.4, also showed no detectable expression of the K^b and K^k-haplotypes of the MHC class I. However, there was significant induction in the expression of MHC class I after treatment with IFN-gamma, as 86% of the treated cells were K^b-haplotype positive and 91% of the treated cells were K^k-haplotype positive. Thus, MOVCAR-2 cells themselves, either from parental cell line or from *in vivo* tumors, did not show significant expression of MHC class I. However, the expression of MHC class I in these cells could be induced by treatment with IFN-gamma. Hence, these results suggest that the MOVCAR-2 cells can be used as a target in the cytotoxicity assays, but only after treatment with IFN-gamma.

Analysis of TAP-1 expression in MOVCAR-2. The MOVCAR-2 cells were also tested for expression of TAP-1 protein before and after treatment with IFN-gamma. The TAP (**T**ransporter **A**ssociated with antigen **P**rocessing) proteins belong to the ABC group of membrane transporters and are responsible for transporting peptides derived from antigens expressed in the cytosol to the endoplasmic reticulum, where they are assembled along with MHC, before being exported to the Golgi apparatus, to be finally inserted into the plasma membrane. Thus, down-modulation in the expression of TAP proteins leads to blockade in the processing and presentation of the tumor antigens on the cell surface. Expression of TAP-1 protein the MOVCAR-2 cells was determined by Western blot analysis. As shown in Fig. 2.5, MOVCAR-2 cells, from parental cell line (lane 1), cells cultured from ascites (lane 3), and cells cultured from solid tumor (lane 5) did not show detectable expression of TAP-1. However, upon treatment with IFN-gamma, the MOVCAR-2 cells from parental cell line (lane 2), cells cultured from ascites (lane 4), and cells cultured from solid tumor (lane 6) showed induction in the expression of TAP-1. The cells from the MOVCAR-2 parental cell line showed greater induction in the expression of TAP-1 than the MOVCAR-2 cells cultured from the ascites and solid tumor upon exposure to IFN-gamma. Thus, like MHC class I, the MOVCAR-2 cells, either from cell line or from tumors, did not show detectable expression of TAP-1. However, the expression was significantly induced upon treatment with IFN-gamma as shown by Western blot analysis. This confirms the results suggesting that MOVCAR-2 cells can be used as target cells for cytotoxicity assays, but only after treatment with IFN-gamma.

Assessment of CTL activity by ^{51}Cr Release Assay. The anti-tumor immune response induced by the CD40-targeted adenoviral vaccine in B6C3F1 mice was quantified by measuring the killing of target cells that express the tumor antigen *in vitro*, by activated tumor antigen-specific cytotoxic T-cells (CTLs) generated *in vivo*. Fig. 2.1 showed that MOVCAR-2 cells do express the target tumor antigen, SV40 T-Ag, and thus can be used as target in the cytotoxicity assay. However, Figs. 2.2, 2.3, 2.4 and 2.5 show that MOVCAR-2 cells can be used as target only after treatment with IFN-gamma, since without exposure to IFN-gamma the cells by themselves, do not express significant amounts of MHC class I and TAP-1, which are necessary for the efficient processing and presentation of the tumor antigen on the cell surface. Thus, IFN-gamma treated MOVCAR-2 cells were chosen as the target cells to measure the lytic activity of the activated antigen-specific CTLs. As shown in Fig. 2.6, the splenocytes isolated from B6C3F1 mice immunized using the CD40-targeted Ad5-CMV-SV40 T-Ag showed increased killing of the target cell line, MOVCAR-2, than the splenocytes isolated from mice immunized using untargeted Ad5-CMV-SV40 T-Ag at all target cell:effector cell (T:E) ratios used. The increase in killing obtained when CD40-targeted adenoviral vector was used was approximately 20% greater than that obtained by untargeted adenovirus at all T:E ratios. Untreated naïve MOVCAR-2 cells that did not express significant levels of MHC class I and TAP-1 was used as a negative control. Likewise, the CMT.64 cell line that does not express SV40 T-Ag, was also used as a negative control. There was minimal killing of both control cell types, with approximately 22 - 24% killing at T:E ratio of 1:100, by the splenocytes isolated from mice immunized using CD40-targeted as well as untargeted Ad5-CMV-SV40 T-Ag. Thus, the chromium release based cytotoxicity assay suggests that CD40-targeted adenovirus expressing tumor antigen can activate antigen-specific T-cells and induce a greater cytotoxic T lymphocyte response than untargeted adenovirus expressing the same antigen.

Assessment of CTL Activity by CFSE-based *in vivo* CTL Assay. The anti-tumor immune response induced by CD40-targeted adenovirus was also determined by measuring the killing of

target cells *in vivo* by activated CTLs using the CFSE-based *in vivo* cytolytic assay. The target cells in this assay were splenocytes pulsed with peptides that correspond to the antigenic epitopes of SV40 T-Ag. Unpulsed splenocytes were used as negative control cells. Both, target and control splenocyte populations were mixed 1:1 and injected intravenously in the B6C3F1 mice immunized using CD40-targeted Ad5-CMV-SV40 T-Ag, 1×10^9 i.f.u. of Ad5-CMV-SV40 T-Ag complexed with 2400 ng of cFm40L and untargeted 1×10^9 i.f.u. of Ad5-CMV-SV40 T-Ag. As shown in Fig. 2.7, mice immunized using CD40-targeted Ad5-CMV-SV40 T-Ag showed similar killing of the target splenocytes as the mice immunized using untargeted Ad5-CMV-SV40 T-Ag. The target cell lysis observed, was approximately between 78-90%. These results were markedly different from those obtained using the *in vitro* CTL assay in which CD40-targeted adenovirus induced a greater cytotoxic T lymphocyte response than untargeted adenovirus expressing the same antigen. These results could be explained by the possibility that the immune system maybe saturated at this high dose, 2 X 10^9 i.f.u. of virus resulting in a lack of difference in between the induced cytotoxic T-cell response between the two groups.

To explore this possibility, lower doses of the virus were used for immunization. The lower doses of Ad5-CMV-SV40 T-Ag used were 2×10^8, 2×10^7, 2×10^6, 2×10^5, 2×10^4, 2×10^3 and 2×10^2 i.f.u. per mouse. The amount of CFm40L complexed with the above doses of virus for targeting to CD40, were 240, 24, 2.4, 0.24, 0.024, 0.0024 and 0.00024 ng, respectively. As shown in Fig. 2.8, the percentage of specific killing obtained by CD40-targeted Ad5-CMV-SV40 T-Ag was similar to that obtained by untargeted Ad5-CMV-SV40 T-Ag at all the doses of virus used. The CD40-targeted Ad5-CMV-GFP (2×10^8 i.f.u. of Ad5-CMV-GFP complexed with 240 ng of CFm40L) and untargeted Ad5-CMV-GFP were also used as controls for immunization. As shown in the representative histograms of Fig.2.9, there was no lysis of the target splenocyte population, that pulsed with peptides corresponding to the antigenic epitopes of SV40 T-Ag (peak M2), in B6C3F1 mice immunized using CD40-targeted Ad5-CMV-GFP and untargeted Ad5-CMV-GFP. In addition, there was no lysis of the control splenocyte population (peak M1) that was not pulsed with any peptide, in mice immunized using CD40-targeted as well as untargeted adenoviral vectors. Thus, these results suggest the killing of target splenocyte population observed in B6C3F1 mice immunized using CD40-targeted and untargeted Ad5-CMV-SV40 T-Ag was mediated by cytotoxic T-cells (CTLs) that are specific for SV40 T-Ag. The results from the CFSE-based *in vivo* cytolytic assay suggest that the cytotoxic T lymphocyte response induced by CD40-targeted Ad5-CMV-SV40 T-Ag was similar to that induced by untargeted Ad5-CMV-SV40 T-Ag.

2.4 Discussion

The result from the Western blot analysis of MOVCAR-2 cells in Fig. 2.1 confirms the expression of the target tumor antigen, SV40 T-Ag, in the parental MOVCAR-2 cell line and also demonstrates the tumors formed *in vivo* by the MOVCAR-2 cells, retain expression of the SV40 T-Ag. It was important to know the expression of the target antigen was retained *in vivo* by the tumor cells because the activated CTLs are specific for target tumor antigen, *i.e.,* they will kill only those cells that express the targeted tumor antigen. Thus, loss or down-modulation of expression of the tumor antigen *in vivo* by tumor cells will render them resistant to killing by the activated antigen-specific lymphocytes. Therefore, the Western blot result (Fig. 2.1) supports the choice of using MOVCAR-2 cells as targets to measure the anti-tumor immune response induced against SV40 T-Ag. The flow cytometric analysis of the target MOVCAR-2 cells, Figs. 2.2, 2.3 and 2.4, reveal that MOVCAR-2 cells do not express significant amount of MHC class I. In fact,

the level of expression, which was at least between 10 - 12% in the parental MOVCAR-2 cell line, decreases to undetectable levels in tumor cells *in vivo*. Thus, loss or down-modulation of MHC expression may be one of the mechanisms by which the MOVCAR-2 cells evade the immune system and form tumors. However, fortunately the expression of MHC class I in MOVCAR-2 parental cell line as well as tumor cells isolated from ascites and solid tumors can be up-regulated significantly by treatment with IFN-gamma as shown in Figs. 2.2, 2.3 and 2.4. This demonstrated the loss or decrease in expression of MHC class I by MOVCAR-2 was because of changes in regulation of the expression and not because of genetic alteration, and the expression can be up-regulated by exposure to inflammatory agents such as IFN-gamma. Likewise, as shown in Fig. 2.5, there was no detectable expression of TAP-1, which is one of the important components of the antigen processing and presentation pathway, in MOVCAR-2 cells. However, this expression was up-regulated by treating the MOVCAR-2 cells with IFN-gamma. Thus, results from Figs. 2.1-2.5 suggest that MOVCAR-2 cells can be used as target cells in cytotoxicity assays to measure the induced anti-tumor CTL response, but only after treatment with IFN-gamma.

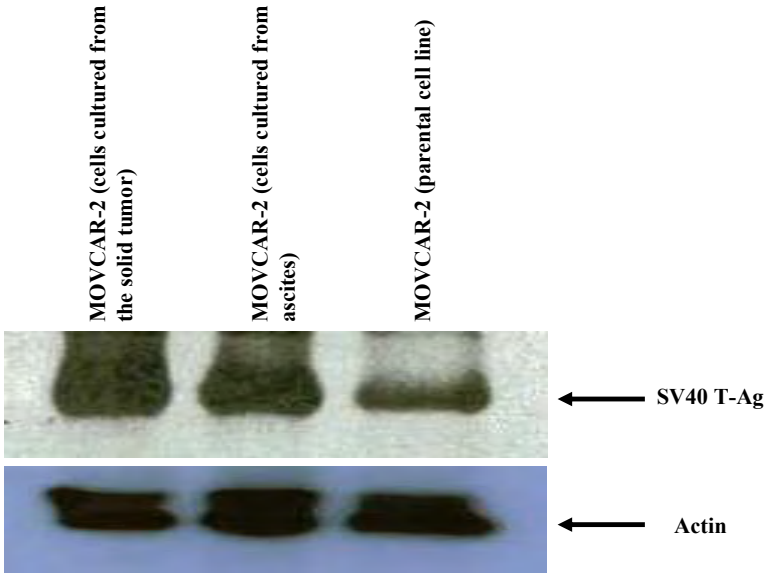
The results from the cytotoxicity assays demonstrate the CD40-targeted adenovirus expressing the tumor antigen SV40 T-Ag does induce an SV40 T-Ag specific cytotoxic T lymphocyte response, *i.e.,* it does induce activation and proliferation of the antigen-specific cytotoxic T lymphocytes *in vivo*. However, the results from the *in vitro* CTL assay differ from that of the *in vivo* CTL assay. The *in vitro* CTL assay, Fig. 2.6, suggests the CTL response induced by the CD40-targeted Ad5-CMV-SV40 T-Ag, as measured by the killing of the target tumor cell line, MOVCAR-2, which expresses SV40 T-Ag, was greater than that induced by untargeted Ad5-CMV-SV40 T-Ag. The CTL response detected was specific for SV40 T-Ag as there was minimal killing of the negative control, cell line CMT.64, which does not express SV40 T-Ag. However, the *in vivo* CTL assay, Figs. 2.7, 2.8 and 2.9, suggest the CTL response induced by the CD40-targeted Ad5-CMV-SV40 T-Ag, as measured by the killing of target splenocytes pulsed with peptides corresponding to antigenic epitopes of SV40 T-Ag, was approximately similar to that induced by the untargeted Ad5-CMV-SV40 T-Ag. Again, the killing activity was specific for cells that express SV40 T-Ag as there was no decrease in the population of the negative control cells, splenocytes that were not pulsed with SV40 T-Ag peptides. In addition, there was no killing of splenocytes observed in mice immunized using CD40-targeted as well as untargeted adenovirus expressing GFP (Ad5-CMV-GFP). Thus although the killing activity observed in both cases was tumor antigen-specific, there was a discrepancy in between the results of the two cytotoxicity assays.

This discrepancy could be because of various factors. The most obvious one is the killing of the target cells takes place *in vitro* in the chromium release assay and *in vivo* in the *in vivo* CTL assay. The difference in the milieu could result in the difference observed in killing. The target cells used in both assays were also different. The *in vitro* CTL assay uses a tumor cell line or primary tumor cells as the target, whereas the *in vivo* CTL assay uses splenocytes as the target cells. The tumor cell line or cells express the tumor antigen endogenously whereas the splenocytes were pulsed *in vitro* with peptides corresponding to the antigenic epitopes of the target tumor antigen. Thus, the tumor cells will express, process and display several epitopes of the tumor antigen on its cell surface, while the splenocytes will only display the epitopes of the antigen that it has been pulsed with. Also, the antigen density on the target cells that are used in both assays is different, and could contribute to the difference in the results obtained from both

assays. Thus, the differences in the results could be attributed to the differences in the experimental protocol and/or the target cells used.

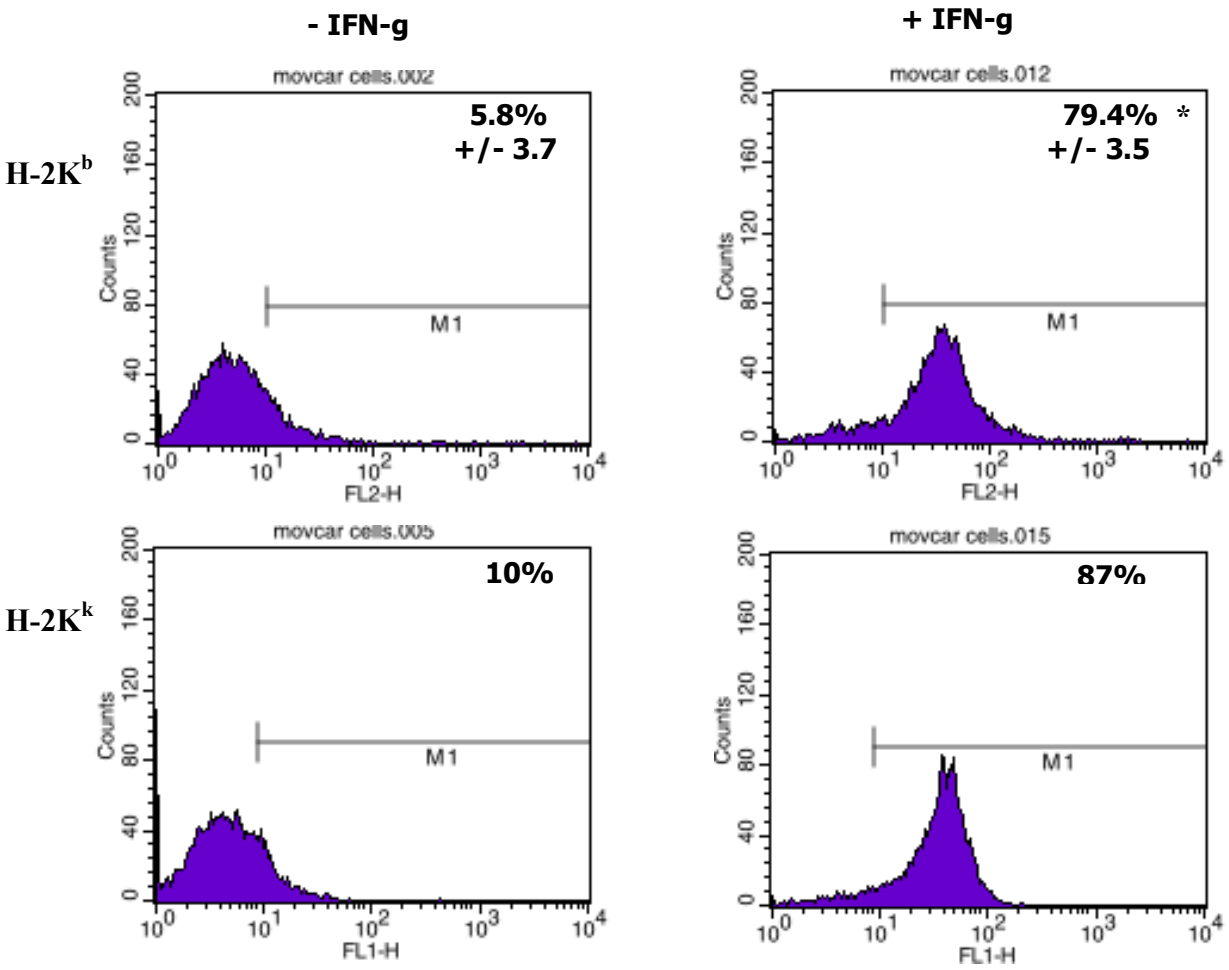
The lytic activity observed using the *in vivo* CTL assay may be more representative of the T-lymphocyte mediated killing of the tumor in animals because of the *in vivo* nature of the assay. In addition, the *in vivo* CTL assay involves injecting both populations of splenocytes, the ones that have not been pulsed with any peptide (negative control) and splenocytes that have been pulsed with peptides corresponding to the target antigen (test), in the same animal and thus there is an internal negative control of the same cellular origin for every mice that was tested for the CTL activity. However, the target cells that were used in the *in vitro* CTL assay, *i.e.,* tumor cell line or tumor cells are the actual target cells that the immune cells will encounter in cancer patients or tumor-bearing animals. Therefore, both assays have their pros and cons. Our hypothesis is that CD40-targeted adenovirus should infect DCs more efficiently than untargeted adenovirus and thus, should be able to elicit a greater CTL immune response than the untargeted vector. Thus, although the results from the *in vitro* chromium release assay were in accordance with our hypothesis, it is difficult to determine which assay is more reliable and to determine whether the CTL response induced by CD40-targeted adenoviral vector is greater than or similar to the CTL response triggered by the untargeted adenovirus. Nonetheless, both assays point the CD40-targeted adenovirus expressing tumor antigen does induce an antigen-specific CTL response, similar, if not greater than that induced by untargeted adenovirus expressing the same tumor antigen. Thus, the CD40-targeted adenoviral vaccine is a potent vaccine.

Figure 2.1
MOVCAR-2 Cells Cultured from Solid Tumor and Ascites Show Expression of SV40 T-Ag



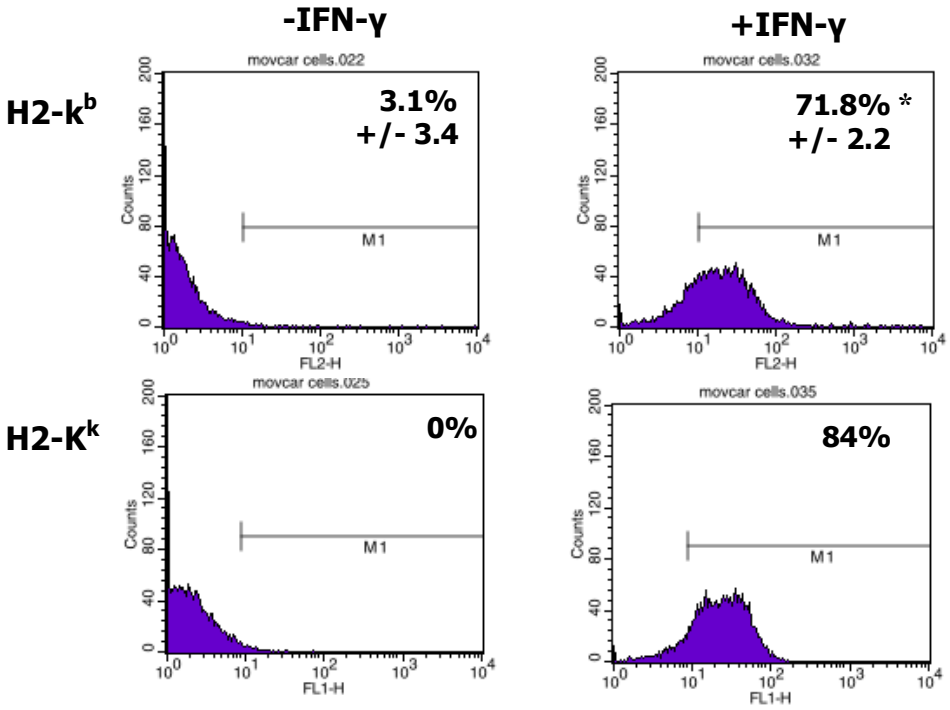
Western blot assay of MOVCAR-2 cells. The expression of SV40 T-Ag was detected in MOVCAR-2 cells cultured from the solid tumor (lane 1), MOVCAR-2 cells cultured from the ascites (lane 2) and the parental MOVCAR-2 cell line.

Figure 2.2
MOVCAR-2 Cell Line Shows an Increase in Expression of MHC-I after Treatment with IFN-γ



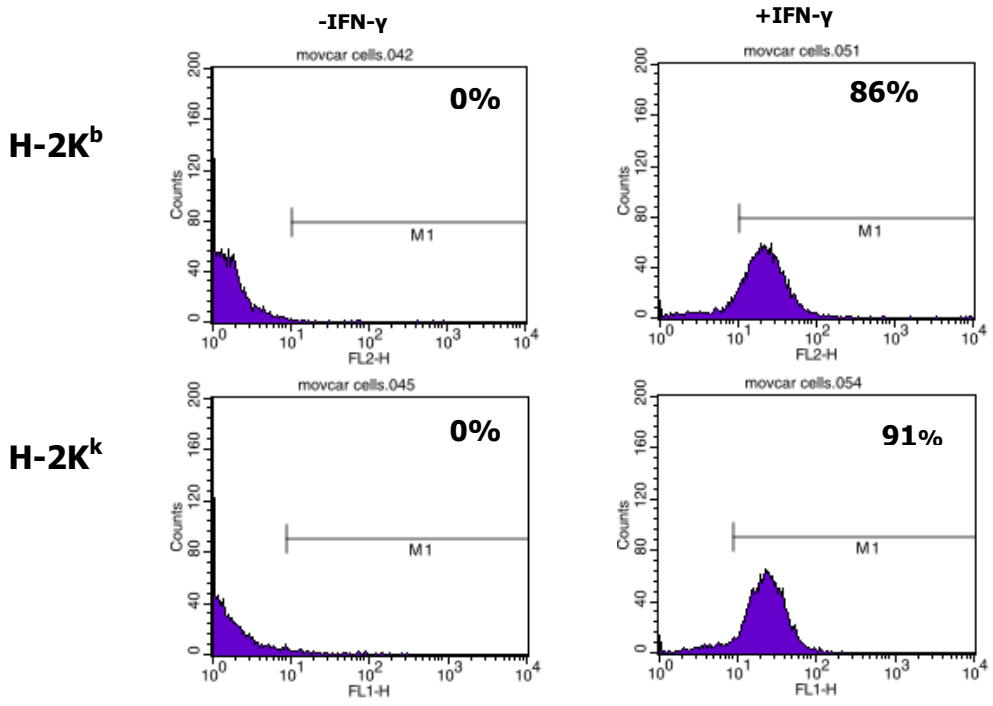
Flow cytometric analysis of the parental MOVCAR-2. The cells were stained with FITC-labeled antibody against MHC class I haplotypes Kb and Kk. * $P < 0.05$ versus control MOVCAR-2 cells, not treated with IFN-g.

Figure 2.3
MOVCAR-2 Cells cultured from Ascites show an Increase in Expression of MHC-I after Treatment with IFN-γ



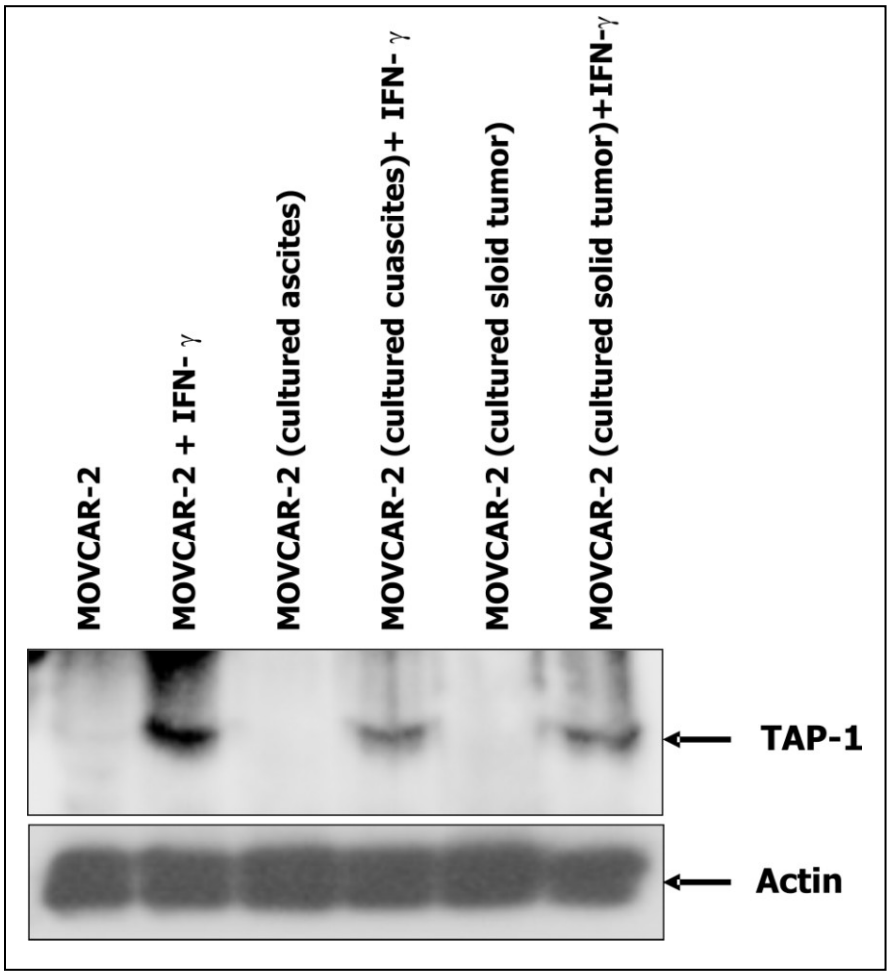
Flow cytometric analysis of MOVCAR-2 cells cultured from the ascites. The cells were stained with FITC-labeled antibody against MHC class I haplotypes Kb and Kk. * $P < 0.05$ versus control MOVCAR-2 cells, not treated with IFN-g.

Figure 2.4
MOVCAR-2 Cells cultured from Solid tumor show an increase in expression of MHC-I after Treatment with IFN-γ



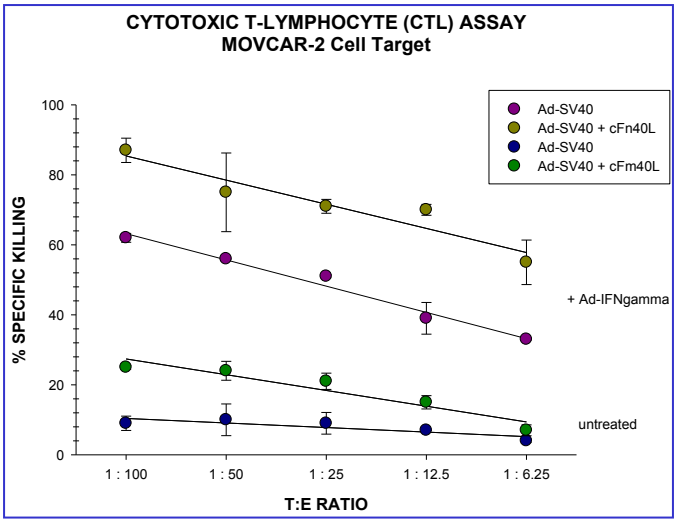
Flow cytometric analysis of MOVCAR-2 cultured from the solid tumor. The cells were stained with FITC-labeled antibody against MHC class I haplotypes Kb and Kk. * $P < 0.05$ versus control MOVCAR-2 cells, not treated with IFN-g.

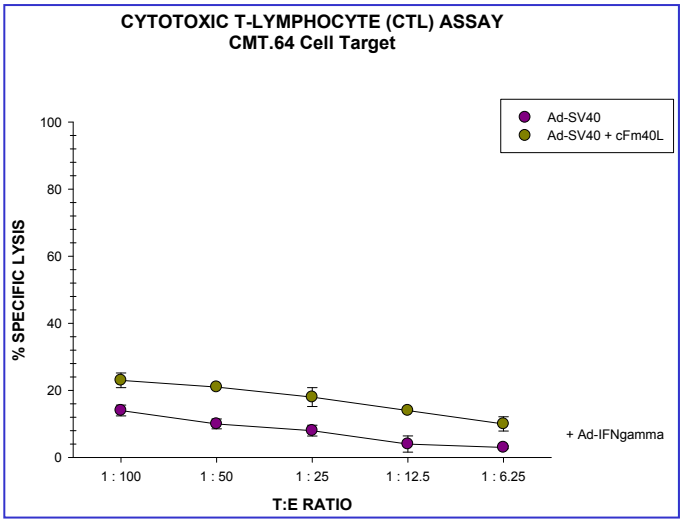
Figure 2.5
MOVCAR-2 Cells Shows an Increase in Expression of TAP-1 after Treatment with IFN-γ



Western blot assay of MOVCAR-2 cells before and after treatment with IFN-gamma. The expression of TAP-1 was detected in MOVCAR-2 cells only after treatment with IFN-gamma. The IFN-gamma treated parental MOVCAR-2 cell line (lane 2), MOVCAR-2 cells cultured from the ascites (lane 4), MOVCAR-2 cells cultured from the solid tumor (lane 6) show expression of TAP-1, but not the untreated MOVCAR-2 cells (lanes 1, 3 and 5).

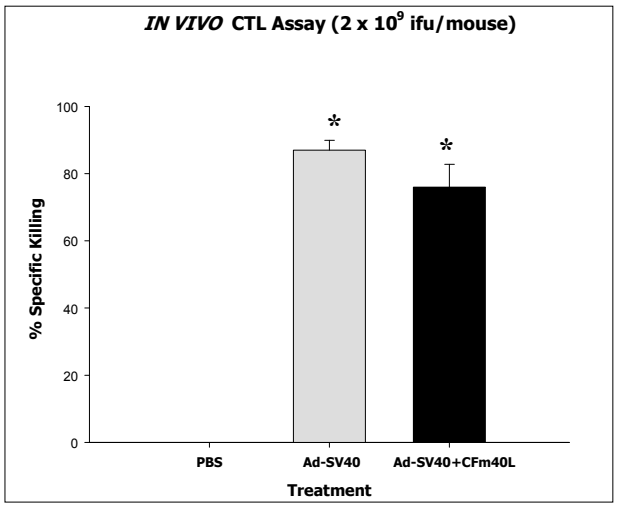
Figure 2.6
CTLs Generated in Mice Immunized using CD40-Targeted Ad-CMV-SV40 T-Ag Show Increased Killing In Vitro of Target MOVCAR-2 Tumor Cells





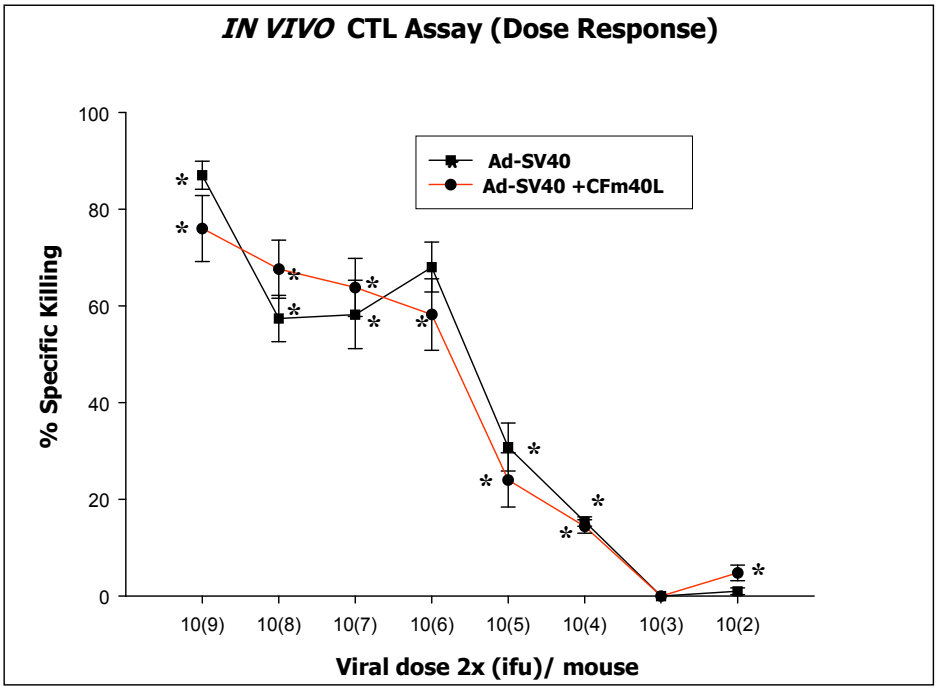
A standard 4 h ^{51}Cr release assay against target cells, MOVCAR-2 (IFN-γ treated as well as untreated) and negative control cells, CMT.64 (IFN-γ treated). The T-cells that were obtained from mice that were treated with Ad5-CMV-SV40 T-Ag + CFm40L showed higher cytolytic activity against the target MOVCAR-2 cells compared with the T-cells obtained from mice that were treated with Ad5-CMV-SV40 T-Ag alone. Each bar represents the mean ± standard error.

Figure 2.7
CTLs generated in mice immunized using CD40-targeted Ad-CMV-SV40 T-Ag show *in vivo* killing of target splenocytes pulsed with peptides corresponding to antigenic epitopes of SV40 T-Ag



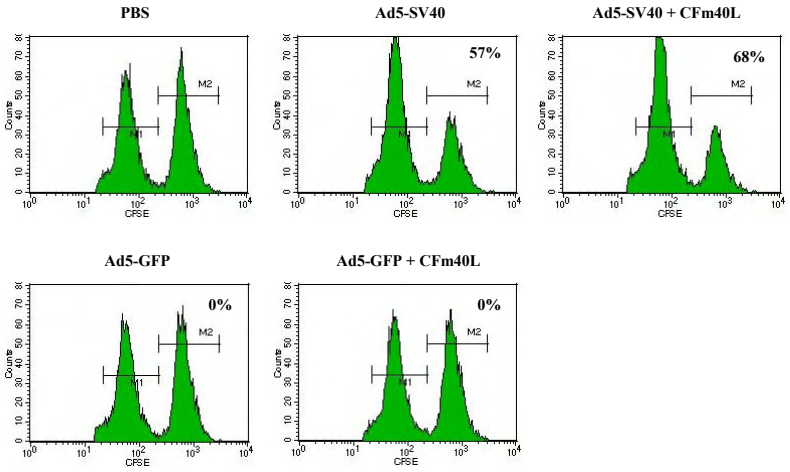
CFSE-based *in vivo* cytotoxicity assay. CD40-targeted Ad5-CMV-SV40 T-Ag and untargeted Ad5-CMV-SV40 T-Ag vaccines induce similar SV40 T-Ag specific cytotoxic T lymphocyte activity. To prepare target cells for detection of *in vivo* cytolytic activity, erythrocytes were removed from naïve B6C3F1 spleen suspensions by osmotic lysis. The cells were then washed and split into two populations. One population was pulsed with 5 μM CKG and 5 μM VVY peptide, incubated at 37°C for 90 min, and labeled with a high concentration of CFSE (5 μM) (CFSEhigh cells). The second control target population was left unpulsed and labeled with a low concentration of CFSE (0.5 μM) (CFSElow cells). For i.v. injection, an equal number of cells from each population was mixed together such that each mouse received a total of 2 X 10^7 cells in 500 μl of PBS. The target cells were then injected into mice that had previously been immunized using CD40-targeted Ad5-CMV-SV40 T-Ag (2 X 10^9 i.f.u. of virus complexed with 2400 ng of CFm40L) and untargeted Ad5-CMV-SV40 T-Ag (2 X 10^9 i.f.u. of virus). Five h after i.v. injection of target cells, mice were sacrificed and harvested of their spleens. Cell suspensions were analyzed by flow cytometry, and each population was detected by their differential CFSE fluorescence intensities. Up to 5 X 10^3 CFSE-positive cells were collected for analysis. To calculate specific lysis, the following formula was used: ratio = (percentage $CFSE^{low}$/percentage $CFSE^{high}$). Percentage of specific lysis = [1 - (ratio unprimed/ratio primed)] X 100. The bars represent the mean values ± standard error of results obtained from 5 mice per treatment. * $P < 0.05$ versus control mice that received only PBS.

Figure 2.8
CTLs generated in mice immunized using CD40-targeted Ad-CMV-SV40 T-Ag show similar *in vivo* killing of target splenocytes as mice immunized using untargeted Ad-CMV-SV40 T-Ag



CFSE-based *in vivo* cytotoxicity assay. CD40-targeted Ad5-CMV-SV40 T-Ag and untargeted Ad5-CMV-SV40 T-Ag vaccines induce similar SV40 T-Ag specific cytotoxic T lymphocyte activity at all the doses of virus that were used for immunization. The B6C3F1 mice were immunized using various doses of CD40-targeted and untargeted Ad5-CMV-SV40 T-Ag, as described in materials and methods. The labeled target and control splenocyte cells, mixed 1:1, were injected intravenously in the immunized mice. After 5 h, the mice were sacrificed, spleens were harvested, and the two populations of splenocytes were analyzed by flow cytometry based on their differential labeling. The percentage of specific killing was also calculated as described in materials and methods. The symbols ● and ■ represent the mean value of percentage specific killing obtained in B6C3F1 mice immunized using CD40-targeted and untargeted Ad5-CMV-SV40 T-Ag respectively. Five mice were used per treatment for each dose of virus used for immunization. Each bar represents the mean ± standard error. * $P < 0.05$ versus control mice that received only PBS.

Figure 2.9
CTLs generated in mice immunized using CD40-targeted Ad-CMV-SV40 T-Ag show *in vivo* killing of target splenocytes pulsed with peptides corresponding to antigenic epitopes of SV40 T-Ag



Representative histograms from the spleens of mice immunized using PBS, Ad5-CMV-SV40 T-Ag and Ad5-CMV-SV40 T-Ag complexed with CFm40L, Ad5-CMV-GFP and Ad5-CMV-GFP complexed with CFm40L to determine the *in vivo* killing of CFSE-labeled target cells. The B6C3F1 mice were immunized using 1 X 10^8 i.f.u. of the above viruses and 240 ng of CFm40L, as described in materials and methods. The target cells were splenocytes harvested from naïve B6C3F1 mice that were pulsed with antigens corresponding to the antigenic epitopes of SV40 T-Ag and labeled with 5 μM of the dye CFSE. Non-pulsed splenocytes from the syngeneic B6C3F1 mice were labeled with 0.5 μM of the same dye CFSE and used as negative control. Both cell types were mixed 1:1 and injected intravenously in the immunized B6C3F1 mice. After 5 h the spleens were harvested from these mice and the splenocytes were analyzed by flow cytometry. The percentage of target and control cells was determined based on their differential labeling intensities. Up to 5 X 10^3 CFSE-positive cells were collected for analysis. Each peak M1 represents the control non-pulsed splenocytes and peak M2 represents the splenocytes pulsed with peptides CKG and VVY that correspond to the antigenic epitopes of SV40 T-Ag.

3. LIVER TOXICITY AND BIODISTRIBUTION STUDIES

3.1 Introduction

Liver toxicity is one of the main obstacles in the use of first-generation adenoviral vectors for application as therapeutic vectors in gene therapy. Ad5 is one of the most commonly used vectors in gene therapy. The natural tropism of wild-type Ad5 is for the liver and thus systemic administration of this virus leads to its accumulation in the liver, producing pathogenic effects in the liver*[15]*. The host immune response to adenovirus is responsible for mediating the toxic effects in the liver*[16, 17]*.

The immune response to adenovirus can be divided into two phases*[18]*. The first phase occurs between day 1 and 4 after infection. It is characterized by an acute inflammatory response which involves the release of certain inflammatory cytokines (IL-6, TNF-α, IL-8, *etc.*) and the recruitment of immune effector cells in the liver. Chemokine expression is induced in the liver within one h after infection and is responsible for the recruitment of neutrophils. The recruitment of neutrophils and other immune effector cells is responsible for mediating the hepatic injury. The second phase begins between 5 to 7 days after infection. The second phase is characterized by an adaptive immune response specific for viral or transgene products. The initial innate and the subsequent adaptive immune response to adenoviral or transgene products are responsible for inducing the toxic effects in the liver.

The sequestration of the adenovirus particles in the liver leads to hepatotoxicity and, the effective dose of adenovirus that can enter the systemic circulation and find its target cell is decreased, reducing the therapeutic effect. The approaches adopted to reduce adenovirus induced toxicity include attempts to reduce the immunogenicity of the virus and to de-target virus from the liver. The attempts to reduce immunogenicity of adenovirus vectors include: 1) deletion of viral genes to reduce the number of viral proteins synthesized*[19-21]*, *e.g.,* gutless adenovirus, and 2) modification of the capsid proteins, genetic as well as non-genetic, such that they are less immunogenic*[22]*. The approaches used to de-target the adenovirus from the liver are: 1) modification, both genetic as well as non-genetic, of the fiber or fiber knob domain, such that it does not bind to CAR *[23, 24]*, and 2) use of bi-specific adapter molecules*[25]*.

Bi-specific adapter molecules can be chemically conjugated targeting moieties or recombinant fusion proteins that can bind to adenovirus fiber knob on one side and to the target cell on the other, serving as a bridge between the adenovirus and the target cell. The use of bi-specific adapter molecule generates adenoviruses which are de-targeted from the liver as the fiber knob domain of the virus is blocked by the adapter molecule. Genetic modification of adenovirus may reduce its production capacity. Non-genetic modification, such as PEGylation*[24]* can reduce the uptake of the virus by the liver, but does not increase its targeting to the target cell. Thus, the use of bi-specific molecules appear to be the most efficient, safe, viable and cost-effective approach to generate targeted adenovirus.

We generated a dendritic cell targeted adenoviral vector in our laboratory by using a bi-specific adapter molecule called CFm40L. This molecule has the CAR domain on one end and the CD40 binding ligand domain on the other end. This molecule can bind to the fiber knob of the adenovirus by its CAR domain. It can also bind to all cell types expressing the CD40 receptor by its CD40-binding ligand domain. Dendritic cells express CD40 receptor on their cell surface. Thus, when the adenovirus and the CFm40L adapter molecule are mixed together, the adapter molecule binds to the adenovirus by its CAR domain. The adenovirus that is coated by

the CFm40L adapter molecule, can infect any cell that expresses CD40 receptor, which includes dendritic cells.

We expected the use of CFm40L adapter molecule will de-target the adenovirus from the liver and re-target it to dendritic cells which express CD40. De-targeting from the liver would lead to reduced liver toxicity and thus more efficient and safer use of adenovirus for immunotherapy. We checked the de-targeting of CD40-targeted adenovirus from the liver by measuring the amount of adenoviral DNA in the livers of mice, after immunization with the virus. We also determined expression of the transgene in the livers of mice after immunization. We also looked for secretion of the liver toxicity enzyme, alanine aminotransferase (ALT), and inflammatory cytokines in the serum of the immunized mice, and performed histological studies of the liver after immunization.

3.2 Materials and Methods

Quantitative Real-Time PCR. B6C3F1 mice were immunized using Ad5-CMV-SV40 T-Ag or Ad5-CMV-SV40 T-Ag complexed with CFm40L as described above. One day after the second immunization the mice were sacrificed and the liver, spleen, and kidney tissues were removed. Total DNA was extracted from 50 mg of liver and kidney and 20 mg of spleen using the QIAamp DNA mini kit (Valencia, CA) according to the manufacturer's instructions. Quantification of adenoviral DNA was performed using Real-Time PCR for E4 gene. The primers and probe sequences were as follows:

forward primer sequence
5'-GGAGTGCGCCGAGACAAC-3',
reverse primer sequence
5'-ACTACGTCCGGCGTTCCAT-3',
probe sequence
5'-6FAM-TGGCATGACACTACGACCAACACGATCT-TAMRA-3'.

The probe was labeled with a reporter fluorescent dye (6-carboxy-fluorescein) at the 5' end and a quencher fluorescent dye TAMRA (6-carboxy-tetramethyl-rhodamine) at the 3' end. Samples were equalized for DNA input using standard primers against mouse GAPDH. The real-time PCR reactions were performed in 50 μl volumes containing 500 ng of total DNA, 100 μM forward primer, 100 μM reverse primer, 50 μM probe and 1X PCR Master Mix (EuroGentec). The following thermal cycling parameters were optimized for adenoviral E4 gene quantification: 2 min at 50°C, 10 min at 95°C, followed by 40 cycles of 1 min at 95°C and 1 min at 60°C.

***Bio-Plex* cytokine assay**. The serum samples were collected from immunized mice one day after the second immunization. The concentrations of Th-2 type cytokines such as IL-5 and IL-13 were analyzed by the *Bio-Plex* multiplex mouse cytokine assay kit (Bio-Rad laboratories, Hercules, CA) and the cytokine reagent kit (Bio-Rad) according to the manufacturer's protocol. Briefly, 50 μl of diluted standards or serum samples were added to a 96-well plate coated with beads that have antibodies specific for the above cytokines conjugated to them. Thus, the cytokines present in the serum will bind to the antibodies that are coupled to the beads. The reaction was incubated at RT by vortexing at 300 rpm for 30 min. The plate was then washed thrice to remove any unbound protein. A mixture of biotinylated antibodies specific for a different epitope on the cytokines was then added to the beads. The reaction was incubated again at RT by vortexing at 300 rpm for 30 min After three washes, Streptavidin-phycoerythrin (streptavidin-PE) was added to the reaction containing biotinylated antibodies bound to the

cytokines in a sandwich. The reaction was incubated at RT by vortexing at 300 rpm for 10 min. After three washes, the data from the reaction was collected and analyzed using the *Bio-Plex* suspension array system (Bio-Rad, Hercules, CA).

Histological examination of liver. B6C3F1 mice were immunized using Ad5-CMV-SV40 T-Ag or Ad5-CMV-SV40 T-Ag complexed with CFm40L as described above. One day after the second immunization, the mice were sacrificed and the livers were removed. The liver tissues were immersion-fixed in 4% buffered formaldehyde, embedded in paraffin, sectioned, and stained with hematoxylin-eosin and Romanoswki's stain.

Analysis of Serum Alanine aminotransferase (ALT) levels. The quantification of ALT levels in the mice serum was done by Charles River Laboratories using ELISA.

MicroPET Analysis of Adenovirus Biodistribution. The B6C3F1 mice were immunized using Ad5-CMV-TK complexed with CFm40L and Ad5-CMV-TK alone. Each mice was injected with 10^9 i.f.u. of Ad5-CMV-TK alone and complexed with 120 ng of CFm40L. Two days after the immunization, the mice were injected in the tail vein with 150 μCi of [^{18}F]-FHBG. After 60 min, the animals were imaged in a microPET scanner, and image data were acquired for 15 min. MicroPET images were reconstructed by using an iterative reconstruction technique.

3.3 Results

Quantification of adenovirus DNA in livers of mice immunized using CD40-targeted and untargeted Ad5-CMV-SV40 T-Ag by Real-time PCR analysis. Binding of the targeting ligand, CFm40L, to the adenovirus masks the CAR-binding fiber knob domain of the adenovirus and targets the virus to cells expressing CD40. Since the CD40-targeted adenoviral vector was retargeted away from cells expressing CAR to cells expressing CD40, we hypothesized the natural liver tropism of the adenovirus would be altered. The potential of CD40-targeted adenovirus to target and infect the liver cells was tested by determining the amount of adenoviral DNA present in the livers of B6C3F1 mice injected with either CD40-targeted or untargeted adenovirus. The adenovirus E4 gene copy number, a surrogate marker for adenovirus infectivity, was quantified by real-time PCR in the livers of the immunized mice. As shown in Fig. 3.1, there was a 6-fold decrease in adenoviral E4 DNA in the livers of mice immunized using the CD40-targeted Ad5-CMV-SV40 T-Ag than in livers of mice immunized using untargeted Ad5-CMV-SV40 T-Ag. Thus, CD40-targeted Ad5-CMV-SV40 T-Ag showed reduced liver infection than untargeted Ad5-CMV-SV40 T-Ag.

Quantification of transgene expression in livers of mice immunized using CD40-targeted and untargeted adenovirus by microPET scan analysis. The results from Fig. 3.1 demonstrated that CD40 targeting of an adenovirus resulted in decreased liver infection than untargeted adenovirus. To confirm this approach of targeting an adenovirus and limiting liver infectivity, HSV-Tk expression was examined by microPET scanning, using [^{18}F]-FHBG as a substrate. In the experiment shown in Fig. 3.2, three mice were imaged as described in the 'Materials and Methods' section. Image data from the control mouse injected i.p. with 0.5 ml saline (A) showed background accumulation of the [^{18}F]-FHBG substrate in the large intestine as well as urinary excretion in the bladder. Clinical toxicity of the Ad vector was primarily hepatic, based on the tropism of the virus to the liver. Intraperitoneal injection of a mouse with 1×10^9 i.f.u. of untargeted Ad-CMV-HSV-Tk resulted in strong expression of HSV-Tk in the liver as demonstrated by accumulation of the [^{18}F]-FHBG (B). Importantly, intraperitoneal injection of a mouse with 1×10^9 i.f.u. of DC40-targeted Ad-CMV-HSV-Tk showed almost no expression in the liver (C). These results clearly show a differential expression between untargeted Ad-CMV-

HSV-Tk and CD40-targeted Ad-CMV-HSV-Tk in normal liver, and demonstrate that CD40 targeting can reduce liver infectivity.

Analysis of inflammatory cytokines in the serum of mice immunized using CD40-targeted and untargeted Ad5-CMV-SV40 T-Ag by *Bio-Plex* Cytokine assay. Liver toxicity induced by adenovirus was initiated by an inflammatory response to the adenovirus present in the liver. Thus, adenovirus-induced liver toxicity was accompanied by an increase in the levels of pro-inflammatory cytokines in the blood. Since results from Figs. 3.1 and 3.2 showed that CD40-targeted adenovirus reduced targeting and infection of the liver, we hypothesized there was an accompanying decrease in inflammatory response to the modified virus. The inflammatory response induced by the CD40-targeted adenovirus was determined by analyzing the serum of the immunized mice for the presence of inflammatory cytokines using the Bio-Plex cytokine assay. The results of this assay in Fig. 3.3, showed an increase in the Th-2-type cytokines IL-5 and IL-13 in the serum of mice immunized using untargeted Ad5-CMV-SV40 T-Ag compared with control naïve mice. In addition, there was a 2.5-fold increase in the amount of IL-5 and a 1.6-fold increase in the amount of IL-13 in the serum samples compared with control naïve mice. Importantly however, there was no significant change in the amounts of IL-5 and IL-13 in the serum of the mice immunized using CD40-targeted Ad5-CMV-SV40 T-Ag compared with control naïve mice or with mice immunized using CFm40L targeting ligand alone. Thus, the amount of the Th-2-type cytokines, IL-5 and IL-13, present in the sera of the mice immunized using CD40-targeted Ad5-CMV-SV40 T-Ag was comparable to that present in the control naïve mice. These results show reduced inflammation in mice immunized using CD40-targeted Ad5-CMV-SV40 T-Ag than mice that received the untargeted Ad5-CMV-SV40 T-Ag.

Histological examination of Liver tissue from mice immunized using CD40-targeted Ad5-CMV-SV40 T-Ag and untargeted Ad5-CMV-SV40 T-Ag. The results from Fig. 3.3 showed there was decrease in the amount of Th-2-type inflammatory cytokines, IL-5 and IL-13, in the sera of mice immunized using CD40-targeted Ad5-CMV-SV40 T-Ag compared with the mice immunized using untargeted Ad5-CMV-SV40 T-Ag. Both IL-5 and IL-13, have been shown to induce inflammation by increasing infiltration of neutrophils and eosinophils. To determine if up-regulation in the amounts of IL-5 and IL-13 led to increased local inflammation, histological examination of the livers of mice immunized using CD40-targeted Ad5-CMV-SV40 T-Ag and untargeted Ad5-CMV-SV40 T-Ag was performed by using hematoxylin and eosin and Romanowski staining. As shown in Fig. 3.4, the hematoxylin and eosin staining of liver sections revealed increased infiltration of immune cells in the livers of mice immunized using untargeted Ad5-CMV-SV40 T-Ag than the control naïve mice. The livers of mice immunized using CD40-targeted Ad5-CMV-SV40 T-Ag showed an absence of infiltration by immune cells, similar to control naïve mice. The Romanowski staining of the liver sections revealed there was increased inflammation of the capsule of the liver (capsulitis), observed in the livers of the mice immunized using the untargeted Ad5-CMV-SV40 T-Ag compared with the livers of the control naïve mice. There was also increased infiltration of leukocytes and lymphocytes in the capsule and in the peri-sinusoidal spaces of the livers of mice immunized using untargeted Ad5 SV450 T-Ag. The livers of mice that received CD40-targeted Ad5-CMV-SV40 T-Ag showed a normal capsule and little infiltration of leukocytes and lymophocytes. Thus, these results demonstrate there was reduced inflammation in the livers of mice immunized using CD40-targeted Ad5-CMV-SV40 T-Ag than mice immunized using untargeted Ad5-CMV-SV40 T-Ag.

Analysis of Alanine Aminotransferase (ALT), in serum of mice immunized using CD40-targeted and untargeted Ad5-CMV-SV40 T-Ag. Another test for determining liver toxicity assessment of serum ALT levels. ALT is an enzyme that plays a role in protein metabolism, and is found mainly in the liver, but also in smaller amounts in the kidneys, heart, muscles, and pancreas. ALT is measured clinically to determine if the liver is damaged or diseased. Low levels of ALT are normally found in the blood. However, when the liver is damaged or diseased, it releases ALT into the bloodstream. Most increases in ALT levels are caused by liver damage. The amount of the liver toxicity enzyme, ALT, in the sera of the immunized mice was measured by ELISA. The results, Fig. 3.5, showed there was an approximately 2.5-fold increase in the amount of the liver toxicity enzyme present in the serum of mice immunized using untargeted Ad5-CMV-SV40 T-Ag compared with control naïve mice. However, the amount of ALT present in the serum of mice immunized using CD40-targeted Ad5-CMV-SV40 T-Ag was similar to the control naïve mice (Figure 3.6). These results demonstrate there was reduced liver toxicity in mice immunized using CD40-targeted Ad5-CMV-SV40 T-Ag compared with the mice immunized using untargeted Ad5-CMV-SV40 T-Ag.

3.4 Discussion

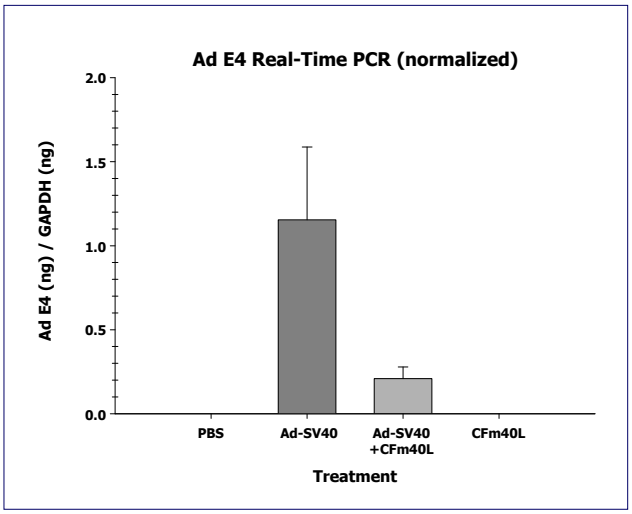
One of the goals in targeting adenovirus was to de-target from the liver, as accumulation of adenoviral particles in the liver induces an acute innate inflammatory response followed by an adaptive immune response to viral proteins and transgenes, which result in liver toxicity*[18]*. Our results show that CD40-targeted adenovirus vector resulted in reduced liver infection and toxicity than the untargeted adenovirus as shown by: 1) decreased amount of adenoviral DNA in the liver, as measured by real-time PCR, 2) reduced expression of the transgene , *thymidine kinase*, in livers of mice injected with CD40-targeted Ad-TK than the mice injected with untargeted Ad-TK, as measured by PET, 3) decreased amount of Th-2-type inflammatory cytokines such as IL-5 and IL-13 in the serum, and 4) reduced infiltration of lymphocytes and leukocytes in the liver. Thus, our results show that by decreasing infection of liver cells, the CD40-targeted adenoviral vector reduced acute inflammatory response and subsequent adaptive immune response, leading to lower liver toxicity.

The Coxsackie and Adenovirus Receptor (CAR), αv-integrins and heparin sulfate glycosaminoglycans and blood factors (Factor IX and C4 Binding Protein) are molecules that bind to adenovirus and determine the tropism of the adenoviral vector *in vivo[26]*. The fiber-knob domain of Ad5 binds to CAR and blood factors. The penton base of the adenoviral particle binds to the αv-integrins. The fiber shaft of the adenovirus binds to Heparin Sulfate Glycosaminoglycans. Studies by Vigne *et al., [27]* suggest that simultaneous ablation of CAR and αv-integrin binding, by introducing mutations in the fiber knob domain and deleting the RGD motif in the penton base respectively, fails to reduce Ad5 liver tropism. While studies by Kim JH *et al., [28]* suggest that ablation of CAR binding, by introducing mutation in the β sheet of the fiber knob, reduces adenovirus liver tropism and toxicity. Our results supports the results of Kim JH *et al.,* and suggests the fiber-knob domain of the adenoviral particle does play an important role in infecting liver cells, as it is this domain that is masked by the CFm40L adapter molecule in the CD40-targeted adenoviral vector. Our findings support the hypothesis that binding of fiber-knob domain to its receptor plays an important role in determining the tropism of Ad5 vector to the liver.

There are various mechanisms of hepatotoxicity*[29]*. Some of them are: 1) Bile acid induced hepatocyte apoptosis, 2) inflammatory liver injury, 3) cytochrome P4502E1 dependent toxicity, 4) peroxynitrite induced hepatotoxicity, and 5) hepatotoxicity due to mitochondrial dysfunction. Our results suggest the mechanism of liver toxicity induced in our study, by use of untargeted adenovirus, is the inflammatory liver injury. Nonetheless, we cannot exclude the other possibilities since we have not tested for them. We saw increased infiltration of leukocytes and lymphocytes in the capsule and around the peri-sinusoidal regions of the liver. There was also increased production of inflammatory cytokines such as IL-5 and IL-13. Both IL5 and IL-13 have been shown to play a role in hepatitis induced liver damage by recruitment of leukocytes in the liver and inducing a Th-2 type immune response (Wynn *et al.)*. Our results support this hypothesis.

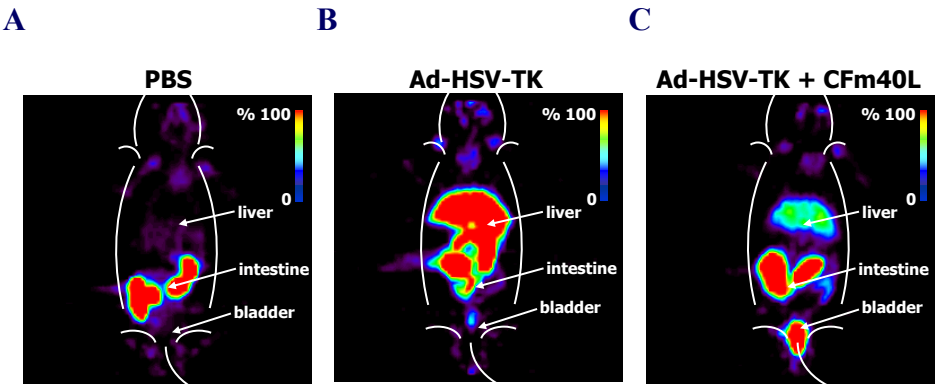
The CFm40L adapter molecule de-targets the adenovirus from the liver, leading to reduced infection of the liver by CD40-targeted adenovirus. The reduced infection of the liver by the virus leads to a decreased immune response, *i.e.,* production of inflammatory cytokines and chemokines and infiltration of the liver by immune cells, and thus reduced liver toxicity. The fact that CFm40L adapter molecule can successfully de-target the adenovirus from the liver also supports the binding between the adapter molecule and adenovirus was stable *in vivo*.

Figure 3.1
Mice immunized using CD40-targeted Ad-CMV-SV40 T-Ag show reduced liver infection



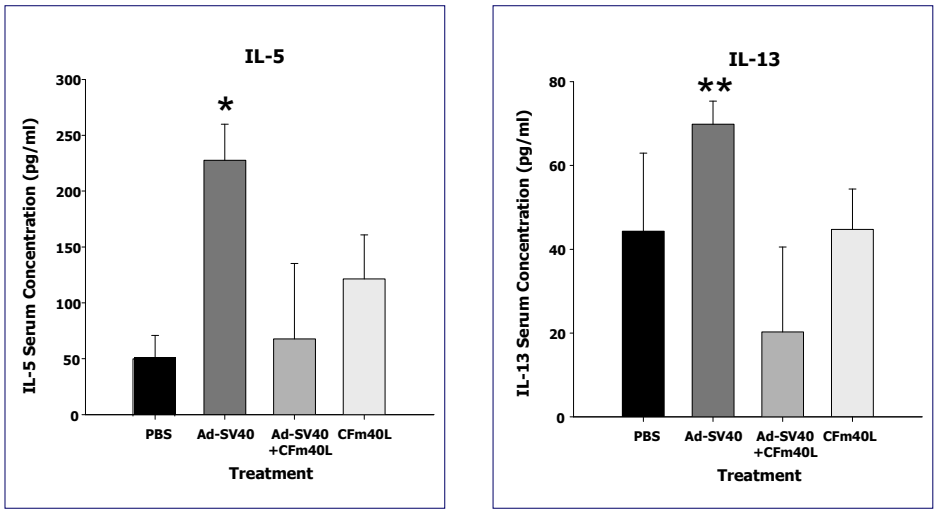
Quantitative comparison of adenoviral E4 DNA in livers of B6C3F1 mice immunized using CD40-targeted Ad5-CMV-SV40 T-Ag and untargeted Ad5-CMV-SV40 T-Ag by TaqMan Real-time PCR. The amount of adenoviral E4 DNA was expressed in nanograms (ng) and was normalized to the amount of mouse GAPDH DNA (ng), present in the liver tissue. There was increased amount of E4 DNA present in the livers of mice immunized using untargeted Ad5-CMV-SV40 T-Ag than the mice immunized using CD40-targeted Ad5-CMV-SV40 T-Ag. n = 5 mice was used per group. Each bar represents the mean ± standard error.

Figure 3.2
Mice immunized using CD40-targeted Ad-CMV-SV40 T-Ag show reduced liver infection



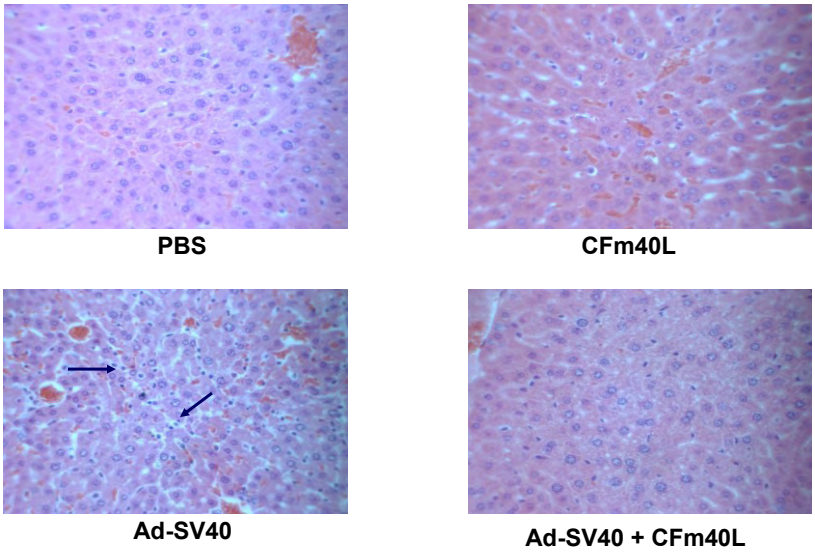
MicroPET imaging illustrating expression of TK in mice injected intraperitoneally with CD40-targeted Ad-HSV-TK and untargeted Ad-HSV-TK. (A) Image data from control mouse that received 500 µl of PBS. (B) Image data from mouse that received 1×10^9 i.f.u. of untargeted Ad-HSV-TK. (C) Image data from mouse that received 1×10^9 i.f.u. of CD40-targeted Ad-HSV-TK. There was higher expression of TK in the livers of mice that received untargeted Ad-HSV-TK than the mice that received CD40-targeted Ad-HSV-TK. The signal bars indicate 0 to 100% activity. The signal bars indicate 0 to 100% relative signal.

Figure 3.3
Mice immunized using CD40-targeted Ad-CMV-SV40 T-Ag show reduced Th-2 Cytokine response



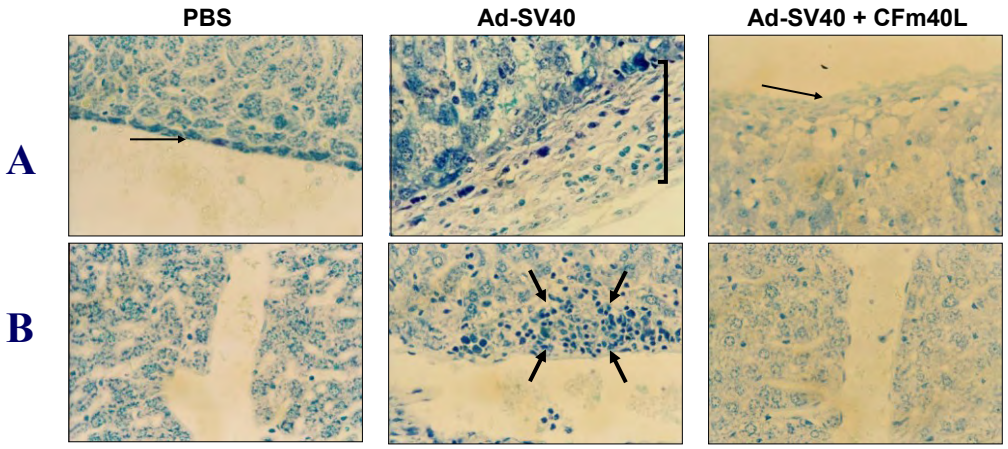
Quantitative analysis of inflammatory cytokines released in the blood of B6C3F1 mice immunized using CD40-targeted Ad5-CMV-SV40 T-Ag, untargeted Ad5-CMV-SV40 T-Ag and CFm40L by the cytokine bead array. Serum samples were collected from the immunized mice and analyzed for presence of cytokines using the *Bio-Plex* cytokine bead array. There was increased amounts of Th-2-type cytokines such as IL-5 and IL-13 in the serum of the mice immunized using untargeted Ad5-CMV-SV40 T-Ag (Ad-SV40) than the mice immunized using CD40-targeted Ad5-CMV-SV40 T-Ag (Ad-SV40 + CFm40L) and the control mice (PBS and CFm40L). n = 3 mice was used per group. Each bar represents the mean ± standard error.
* $P < 0.05$ versus control mice that received only PBS. ** $P < 0.05$ versus mice that were immunized with CD40-targeted Ad5-CMV-SV40 T-Ag (Ad-SV40+CFm40L).

Figure 3.4
Mice immunized using CD40-targeted Ad-CMV-SV40 T-Ag show reduced Liver Inflammation



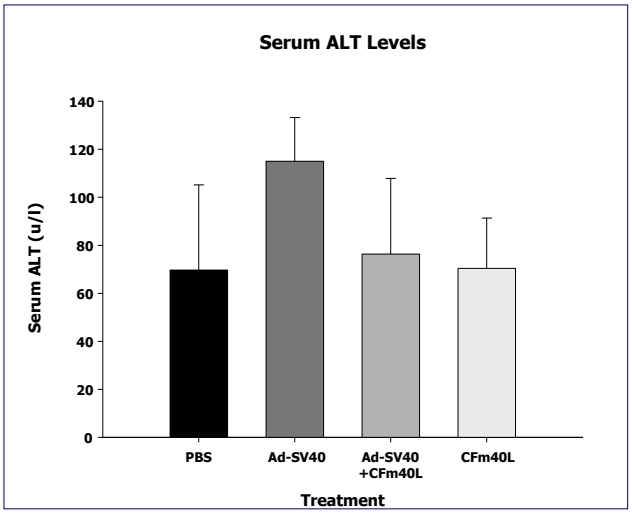
Hematoxylin and Eosin Staining of liver tissue from B6C3F1 mice immunized using CD40-targeted Ad5-CMV-SV40 T-Ag, untargeted Ad5-CMV-SV40 T-Ag and CFm40L. The mice injected with untargeted Ad5-CMV-SV40 T-Ag (Ad-SV40) showed increased infiltration of the liver by mononuclear inflammatory cells compared with livers of mice injected with CD40-targeted Ad5 SV40 Tag (Ad-SV40 + CFm40L) and control treatments (PBS, CFm40L).

Figure 3.5
Mice immunized using CD40-targeted Ad-CMV-SV40 T-Ag show reduced liver inflammation



Romanowski staining of liver tissue from B6C3F1 mice immunized using CD40-targeted Ad5-CMV-SV40 T-Ag and untargeted Ad5-CMV-SV40 T-Ag. The mice injected with untargeted Ad5-CMV-SV40 T-Ag (Ad-SV40) showed increased (A) inflammation of the capsule of the liver and (B) infiltration of the mononuclear inflammatory cells in the peri-sinusoidal spaces of the liver compared with the mice immunized using CD40-targeted Ad5-CMV-SV40 T-Ag (Ad-SV40 + CFm40L) and the control mice (PBS).

Figure 3.6
Mice immunized using CD40-targeted Ad-CMV-SV40 T-Ag show reduced serum ALT response



Analysis of serum marker for Liver damage, alanine aminotransferase (ALT), by ELISA. There was increased levels of ALT in the serum of mice immunized using untargeted Ad5-CMV-SV40 T-Ag (Ad-SV40) compared with the mice immunized using CD40-targeted Ad5-CMV-SV40 T-Ag (Ad-SV40 + CFm40L) and control treatments (PBS, CFm40L). n = 5 mice per group was used. Each bar represents the mean ± standard error.

4. IMMUNIZATION OF MICE AND TUMOR CHALLENGE EXPERIMENTS

4.1 Introduction

The anti-tumor efficacy of any pre-clinical therapy needs to be evaluated using a reliable marker of tumor inhibition. The most relevant marker is reduction in tumor growth and increase in survival of treated animals. Thus, survival studies are the gold standard in evaluating the efficacy of anti-cancer therapies. However, by focusing only on the end-point, one misses out on the changes in tumor growth that occur between. The changes in the kinetics of tumor growth that occur in response to anti-tumor therapy can be monitored by regularly measuring the size of the growing tumors. However, this is only possible with superficially growing tumors and not with tumors that are not superficial, *e.g.,* visceral tumors, metastatic and disseminated tumors. In addition, the small changes in tumor mass are missed, when only the gross tumor dimensions are measured. The changes in tumor mass can be monitored by weighing the animals. However, in the early stages of tumor growth, weight is not a reliable marker since it can be influenced by many other factors. Thus, monitoring the proliferation of the tumor cells *in vivo* with the changes in weight of the animals would be a good approach to monitor tumor progression in the animals.

Bioluminescent imaging was chosen to monitor the growth of tumor cells *in vivo* in our studies. Bioluminescence is the production of visible light during an enzymatic reaction. The enzyme responsible for the production of light is luciferase and the substrate that it acts upon is luciferin. The most commonly used luciferase enzyme and its substrate, luciferin are produced by the firefly *Photinus pyralis*. The enzyme catalyzes the reaction,

[ATP + luciferin + O_2= AMP + oxyluciferin + PPi + light (560 nm)] in the presence of Mg^{2+}. Introducing the firefly *luciferase* gene in mammalian cells can provide a bio-marker to visualize the cells when luciferin is added. When these cells with the *luciferase* gene are injected into animals, they can be visualized *in vivo* when luciferin is injected into the animals. Luciferin is non-toxic to animals. Bioluminescent imaging was chosen to monitor tumor growth as it is a non-invasive approach and it has a greater intensity than fluorescent imaging to image visceral tumors*[30]*.

Ovarian cancer cell lines, IG10 and HM-3, were stably transfected with a plasmid that encoded for the SV40 T-Ag protein, since that is the target tumor antigen against which the immune response is raised. HM-3 is a sub line of OV2944 which is a ovarian tumor cell line of B6C3F1 origin*[31]*. IG10 is a mouse ovarian cancer cell line of C57BL/6 origin*[32]*. Cell lines (IG10 and HM-3) that did not originally express SV40 T-Ag were chosen as they would serve as excellent negative controls, since they would be the same as the target cells, but, without the target SV40 T-Ag. This would provide a good pair of target (IG10-SV40 and HM-3-SV40) and control (IG10 and HM-3) cells, both essentially from the same parental cell line, but one with the target tumor antigen (SV40 T-Ag) and one without. These cells were then stably transfected with a plasmid that encoded for the firefly luciferase so the growth of these cells *in vivo* in B6C3F1 mice can be visualized by imaging. The tumor-forming potential of the transfected cell lines was tested by injecting the cells in B6C3F1 mice, and the rate of tumor growth was monitored by bioluminescent imaging. The status of expression of the components of the antigen processing and presentation pathway, such as TAP-1 and MHC class I, in the transfected cell lines was determined to evaluate the efficacy of these cells to be recognized by the activated tumor antigen-specific lymphocytes.

After evaluation of the tumor cell lines for their tumorigenicity, expression of tumor antigen and MHC-I, the IG10-SV40 cell line were used in tumor challenge experiments. The efficacy of

the protective anti-tumor immunity generated by CD40-targeted and untargeted Ad5-CMV-SV40 T-Ag was evaluated by immunizing the immunocompetent B6C3F1 mice with the respective viral vectors, and challenging them the tumor cells. The challenged mice were then monitored every week for change in body weight and survival.

4.2 Materials and Methods

Cell culture of IG10 and HM-3 cells. The IG10 cells were purchased from the ATCC and maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% Fetal bovine Serum (FBS) (Gemini BioProducts) and 1% antibiotic antimycotic (Invitrogen). The HM-3 mouse ovarian cancer cell line was provided by Dr. K. Yokoro, Department of Biophysics, Faculty of Science, Kyoto University*[31]*. The HM-3 cells were also maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% Fetal bovine Serum (FBS) (Gemini BioProducts) and 1% antibiotic antimycotic (Invitrogen).

Stable transfection of SV40 T-Ag and Luciferase in IG10 and HM-3 cells. The cDNA encoding SV40 T antigen (2600 bp) was cloned into the pZIP-Neo SV(X)1 mammalian expression vector (Addgene, MA). The recombinant plasmid was then stably transfected into IG10 and HM-3 cells using lipofectamine plus^{TM} (Invitrogen, CA). The IG10 and HM-3 cells expressing SV40 T antigen were isolated by selection of the neomycin resistant cells in DMEM media containing 500 μg/ml of neomycin. The cDNA encoding luciferase was cloned into the pSELECT-Hyg expression vector (Invivogen, CA). The recombinant plasmid containing the luciferase gene was stably transfected into IG10 and HM-3 cells expressing SV40 T-Ag as well as parental IG10 and HM-3 cells, using lipofectamine plus^{TM} (Invitrogen, CA). The IG10 and HM-3 cells expressing luciferase, IG10-Luc and HM-3-Luc, were isolated by selection of the hygromycin resistant cells in DMEM media containing 200 μg/ml of hygromycin. The IG10 and HM-3 cells expressing both SV40 T-Ag and luciferase, IG10-SV40-Luc and HM-3-SV40-Luc, were isolated by selection of the neomycin and hygromycin resistant cells in DMEM media containing 500 μg/ml of neomycin and 200 μg/ml of hygromycin.

Analysis of SV40 T-Ag expression in IG10-SV40-Luc and HM-3-SV40-Luc cells. The IG10-SV40-Luc, HM-3-SV40-Luc, IG10-Luc and HM-3-Luc cells were cultured *in vitro*. The cells were harvested when 70% confluency was achieved and lysates were prepared. Protein concentrations of each sample were determined using the Bradford method, and all lysates were normalized to the lowest concentration of protein. Samples were loaded onto 10% SDS-polyacrylamide gels, separated by electrophoresis, and transferred to nitrocellulose membranes (15V for 15 min). Membranes were blocked for 1 h with 5% bovine serum albumin in Tris-Cl buffered saline (TBS), followed by two 5 min washes with 1% Tween-20 in TBS (TTBS). The membranes were incubated for 1 h in TTBS containing a primary mouse anti-mouse SV40 T-Ag antibody (CBD Biosciences clone PAb101), and washed three times for 30 min each with TTBS. Afterwards, the membranes were incubated for 1 h in TTBS containing a horseradish peroxidase-labeled goat anti-mouse IgG antibody, and washed three times for 30 min each with TTBS. Finally the membranes were developed using the ECL substrate (Amersham), and exposed to x-ray film.

Analysis of tumorigenicity of IG10 and HM-3 cells stably transfected with plasmid encoding SV40 T-Ag. IG10-SV40-Luc and IG10-Luc as well as HM-3-SV40-Luc and HM-3-Luc cells were injected in B6C3F1 mice. Three B6C3F1 mice were injected intraperitoneally per cell line at the rate of 1 X 10^7 cells per mouse. The mice were then monitored for tumor growth by measuring the expression of luciferase *in vivo* by bioluminescent imaging of the mice

using the IVIS scan. The mice were scanned weekly until the time they showed obvious tumor growth and had to be sacrificed.

***In vitro* assay of growth curve of HM-3 cells:** HM-3 and HM-3-SV40 cells were plated separately in 100 mm tissue culture dishes.1 X 10^5 cells were plated in each dish. The cells of each cell line were harvested each day after plating, until 7 days and the number of viable cells of each cell type was determined by using the Vi-Cell cell viability analyzer (Beckman Coulter). The Vi-Cell automates the widely accepted trypan blue exclusion method to determine the viability of cells.

Analysis of expression of MHC class I in IG10-Luc, IG10-SV40-Luc, HM-3-Luc and HM-3-SV40-Luc cells by flow cytometry. The cell lines were analyzed for expression of H-2K^b haplotype of MHC class I, on their cell-surface. The cells were also activated by addition of recombinant mouse IFN-gamma (R&D Systems) to measure the induction of MHC class I. After 48 h of incubation *in vitro*, the cells were harvested and stained, and 5 X 10^5 cells of each cell line were stained per well for H-2K^b of MHC class I (BD Biosciences clone AF6-88.5). The antibodies were used at 1 µg/ml. Labeled antibody were added to the cells of interest and incubated in the dark for 20 min at 4^0C. Isotype control antibodies were also used to set up compensation. The cells were fixed in 1% paraformaldehyde for 5 min in the dark, washed twice and resuspended in 200 µl of FACS buffer. The cells were analyzed using a FACScan flow cytometer (Becton Dickinson).

Analysis of expression of TAP 1 in IG10-Luc, IG10-SV40-Luc, HM-3-Luc and HM-3-SV40-Luc cells by Western blot. The cell lines were analyzed for expression of TAP 1. The cells activated by addition of recombinant mouse IFN-gamma (R&D Systems) were used as the positive control. After 48 h of incubation *in vitro*, the cells were harvested and lysates were prepared. Protein concentrations of each sample were determined using the Bradford method, and all lysates were normalized to the lowest concentration of protein. Samples were loaded onto 10% SDS-polyacrylamide gels, separated by electrophoresis, and transferred to nitrocellulose membranes (15V for 15 min). Membranes were blocked for 1 h with 5% bovine serum albumin in Tris-Cl buffered saline (TBS), followed by two 5 min washes with 1% Tween-20 in TBS (TTBS). The membranes were incubated for 1 h in TTBS containing a primary mouse anti-mouse TAP-1 (Santa Cruz Biotechnology, catalog # 11465), and washed three times for 30 min each with TTBS. Afterwards, the membranes were incubated for 1 h in TTBS containing a horseradish peroxidase-labeled goat anti-mouse IgG antibody, and washed three times for 30 min each with TTBS. Finally the membranes were developed using the ECL substrate (Amersham), and exposed to x-ray film.

Analysis of SV40 T-Ag expression in IG10-SV40-Luc and HM-3-SV40-Luc tumor cells by Western blot. IG10-SV40-Luc as well as HM-3-SV40-Luc were injected in B6C3F1 mice. Three B6C3F1 mice were injected intraperitoneally per cell line at the rate of 1 X 10^7 cells per mouse. The mice were then monitored for tumor growth. The mice were sacrificed when they showed obvious tumor growth and ascites as well as solid tumors were collected from these mice. The tumor cells cultured from the ascites and solid tumor collected from B6C3F1 mice injected with the cell lines were analyzed for expression of the tumor antigen, SV40 T-Ag. The cells were harvested and lysates were prepared. Protein concentrations of each sample were determined using the Bradford method, and all lysates were normalized to the lowest concentration of protein. Samples were loaded onto 10% SDS-polyacrylamide gels, separated by electrophoresis, and transferred to nitrocellulose membranes (15V for 15 min). Membranes were blocked for 1 h with 5% bovine serum albumin in Tris-Cl buffered saline (TBS), followed by

two 5 min washes with 1% Tween-20 in TBS (TTBS). The membranes were incubated for 1 h in TTBS containing a primary mouse anti-mouse SV40 T-Ag antibody (BD Biosciences, clone PAb101), and washed three times for 30 min each with TTBS. Afterwards, the membranes were incubated for 1 h in TTBS containing a horseradish peroxidase-labeled goat anti-mouse IgG antibody, and washed three times for 30 min each with TTBS. Finally the membranes were developed using the ECL substrate (Amersham), and exposed to x-ray film.

Prophylactic adenoviral immunization. B6C3F1 mice were immunized with the CD40-targeted and untargeted adenovirus. Each mouse received either 10^8 ifu of Ad5-CMV-SV40 T-Ag complexed with 1200ng of CFm40L or 10^8 ifu of Ad5-CMV-SV40 T-Ag by itself, intraperitoneally. Mice that received only PBS, CD40-targeted Ad5-null (10^8 ifu of virus complexed with 1200 ng of CFm40L per mouse), untargeted Ad5-null (10^8 ifu of virus per mouse) and CFm40L (1200 ng per mouse) were used as controls. The mice received a booster dose of the same immunization two weeks later. 10 mice were used per immunization group. Ten days after the booster immunization, the mice were challenged with tumor cells.

Tumor challenge. The IG10(SV40) cells were activated by addition of recombinant mouse IFN-gamma (R&D Systems). After 48 hr of incubation *in vitro*, the cells were harvested from cell culture flasks using 1% trypsin and washed with PBS. The cells were injected into the B6C3F1 mice immunized as described above. Each mouse received 10^7 cells intraperitonealy at day 10 after the boost immunization.

Survival studies. The tumor challenged mice were monitored weekly for changes in tumor growth. The change in bodyweight of the mice was used as an indicator of tumor growth and was measured every week until 11 weeks after challenge with the tumor cells. Mice that exhibited significant tumor burden or pain and distress were euthanized by asphyxiation with CO_2 according to the guidelines of the Institutional Animal Care and Research Advisory Committee at Louisiana State University Health Sciences Center, Shreveport, LA.

4.3 Results

Tumorigenicity of IG10 cells. The anti-tumor efficacy of the CD40-targeted Ad5-CMV-SV40 T-Ag vaccine can be determined by measuring the growth of tumor cells expressing the SV40 T-Ag *in vivo*. Bioluminescent imaging is one of the ways by which tumor progression can be monitored *in vivo*. IG10 cells stably transfected with plasmids encoding SV40T-Ag and luciferase was used as the target tumor cell since it expresses SV40 T-Ag (Fig. 4.1). IG10 cells stably transfected with plasmid encoding for luciferase was used as negative control since it does not express SV40 T-Ag. To confirm the tumor forming potential of the IG10 cells in B6C3F1 mice and to measure the growth of these tumor cells in the mice, three B6C3F1 mice were injected with IG10 cells expressing luciferase (IG10-Luc) at 5 X 10^7 cells per mouse and *in vivo* bioluminescent imaging of luciferase was used to monitor tumor progression. The mice were scanned for luciferase expression using the IVIS imaging system 24 h after injecting the tumor cells. The mice were then scanned weekly for change in expression of luciferase to monitor the growth of the IG10 tumor cells in B6C3F1 mice. As shown in Fig. 4.2, the mice show marked increase in the expression of luciferase at 1 week post-injection compared with 1 day after injection, indicating increase in growth of the IG10-Luc tumor cells *in vivo*. This is the proof-of-principle that growth of IG10 tumor cells can be monitored by *in vivo* bioluminescent imaging. Survival studies were also done on these animals. The survival time of these non-immunized B6C3F1 mice was approximately 4 weeks post-injection.

Analysis of cell-surface expression of MHC Class I in IG10. Expression of MHC class I on IG10-Luc and IG10-SV40-Luc cells was determined by flow cytometry. These cells were also treated *in vitro* with IFN-gamma to measure the induction of MHC class I after exposure to IFN-gamma. At 48 h after treatment with IFN-gamma, the cells were harvested and analyzed for induction of MHC class I expression on the cell surface. As shown in Fig. 4.3, the MHC class I expression was undetectable by flow cytometry in the naïve IG10-Luc and IG10-SV40-Luc cells. However, the expression of MHC class I increased to 88% in IG10-Luc cells and to 94% in IG10-SV40-Luc cells, when these cells were treated with IFN-gamma. Thus, IG10-Luc and IG10-SV40-Luc cells themselves do not show significant expression of MHC class I. However, the expression of MHC class I in these cells can be induced by treatment with IFN-gamma. Hence, these results suggest that both IG10-SV40-Luc and IG10-Luc cells can be used as target and negative control cells respectively, to measure the induced anti-SV40 T-Ag immune response, but only after treatment with IFN-gamma.

Analysis of TAP-1 expression in IG10. The IG10 cells were also tested for expression of TAP-1 protein before and after treatment with IFN-gamma by Western blot analysis. As shown in Fig. 4.4, IG10-Luc cells (lane 1) and IG10-SV40-Luc cells (lane 3) do not show detectable expression of TAP-1. However, upon treatment with IFN-gamma, both IG10-Luc cells (lane 2) and IG10-SV40-Luc (lane 4) show induction in the expression of TAP-1. Thus, like MHC class I, the IG10-Luc and IG10-SV40-Luc cells, do not show detectable expression of TAP-1. However, the expression was significantly induced upon treatment with IFN-gamma as shown by Western blot analysis. This confirms the results suggesting that IG10-SV40-Luc and IG10-Luc cells can be used as target and negative control cells respectively, to measure the induced anti-SV40 T-Ag immune response, but only after treatment with IFN-gamma.

Analysis of SV40 T-Ag expression in IG10-SV40 tumors. Prior to using IG10-SV40 cells as target cells to measure the induced immune response against SV40 T-Ag, it was important to confirm that the expression of SV40 T-Ag was retained in tumors formed *in vivo*. Thus, to determine expression of SV40 T-Ag, in IG10-SV40 tumors, 5 X 10^7 IG10-SV40 cells were injected i.p. into B6C3F1 mice. The mice were monitored for tumor growth; after approximately 4 weeks, the mice started to show signs of tumor growth and were sacrificed and examined histopathologically. The IG10-SV40 cells formed both ascites as well as solid tumors *in vivo*. The cells from the ascites as well as solid tumors were cultured *in vitro* and tested for the expression of SV40 T-Ag. As shown in Figure 4.5, the tumor cells cultured from both, the ascites (lane 1) and solid tumor (lane 2) showed expression of SV40 T-Ag. Thus, IG10-SV40 cells retain expression of the target tumor antigen, SV40 T-Ag, in tumors *in vivo*.

Analysis of HM-3 cells stably transfected by expression plasmid encoding SV40 T-Ag for expression of SV40 T-Ag. The HM-3 cells were stably transfected with an expression vector encoding the SV40 T-Ag. The expression of SV40 T-Ag in the stably transfected HM-3 cells was confirmed by Western blot analysis. As shown in Fig. 4.6, the HM-3 cells that were stably transfected with the plasmid encoding SV40 T-Ag (lane 2), did show expression of the SV40 T-Ag. The parental HM-3 cells (lane 1) do not express SV40 T-Ag and was used as the negative control. Thus, the stably transfected HM-3-SV40 cells do express the target tumor antigen, *i.e.,* SV40 T-Ag, and thus can be used as target cells to measure the immune response induced against SV40 T-Ag in B6C3F1 mice.

Tumorigenicity of HM-3 cells. The anti-tumor efficacy of the CD40-targeted Ad5-CMV-SV40 T-Ag vaccine in B6C3F1 mice can be determined by measuring the growth of tumor cells expressing the target SV40 T-Ag in immunized mice. HM-3 cells stably transfected with

plasmids encoding SV40T-Ag and luciferase, HM-3-SV40-Luc, were used as the target tumor cells since they expresses SV40 T-Ag (Fig. 4.6). HM-3 cells stably transfected with plasmid encoding for luciferase only, HM-3-Luc, were used as negative control since they do not express SV40 T-Ag. To confirm the tumor-forming potential of HM3(SV40)-Luc and HM-3-Lu cells, three B6C3F1 mice were injected with HM-3-SV40-Luc cells and three mice were injected with HM-3-Luc cells at 5 X 10^7 cells per mouse and *in vivo* bioluminescent imaging of luciferase was used to monitor tumor progression. The mice were scanned for luciferase expression using the IVIS imaging system 24 h after injecting the tumor cells. The mice were then scanned weekly for change in expression of luciferase to monitor the growth of the HM-3-SV40-Luc and HM-3-Luc tumor cells in B6C3F1 mice.

At 4 weeks post-injection, the mice injected with HM-3-SV40-Luc cells showed tumor growth and as shown in Fig. 4.7, they also showed marked increase in the expression of luciferase compared with 1day after injection with tumor cells. At 7 weeks post-injection, the B6C3F1 mice injected with HM-3-Luc cells showed signs of tumor growth and also, as shown in Fig. 4.8, increased expression of luciferase compared with day 1 after injection with the tumor cells. This is the proof-of-principle that growth of HM-3-SV40 and HM-3 tumor cells in B6C3F1 mice can be monitored by *in vivo* bioluminescent imaging.

Determination of growth curve of HM-3 cells *in vitro*. The *in vivo* bioluminescent imaging of HM-3 cells in B6C3F1 mice suggested that HM-3-SV40 cells grow and multiply faster than HM-3 cells. To confirm this observation, an *in vitro* assay to determine the growth curve of the HM-3 cells was done. 1 X 10^5 HM-3 and HM-3-SV40 cells were plated in 100 mm Petri-dishes. The cells were harvested and the number of viable cells of each cell line was counted everyday until 7 days after plating the cells. The growth curve of each cell line was plotted by plotting the total number of viable cells obtained on each day after seeding, against the number of days after plating, at which the cells were counted. The growth curve of HM-3-SV40 cells was higher than the growth curve of HM-3 cells, indicating that HM-3-SV40 proliferates faster than the HM-3 cells. The difference in between the growth rates of the two cell lines was most significant at 6 days after plating the cells, when the total number of viable HM-3-SV40 cells was approximately 7.5 X 10^6, while it was approximately 3.0 X 10^6 for HM-3 cells.

Analysis of cell-surface expression of MHC Class I in HM-3. Expression of MHC class I on HM-3-Luc and HM-3-SV40-Luc cells was determined by flow cytometry. These cells were also treated *in vitro* with IFN-gamma to measure the induction of MHC class I after exposure to IFN-gamma. At 48 h after treatment with IFN-gamma, the cells were harvested and analyzed for induction of MHC class I expression on the cell surface. As shown in Fig. 4.10, the MHC class I expression was extremely low in the naïve cells. 1.7 % of HM-3-Luc was MHC class I-positive and 2.3 % of HM-3-SV40-Luc cells were MHC class I-positive before IFN-gamma treatment. However, the expression of MHC class I increased to 88% in HM-3-Luc cells and to 94% in HM-3-SV40-Luc cells, when these cells were treated with IFN-gamma. Thus, HM-3-Luc and HM-3-SV40-Luc cells themselves show insignificant expression of MHC class I. However, the expression of MHC class I in these cells can be induced by treatment with IFN-gamma. Hence, these results suggest that both HM-3-SV40-Luc and HM-3-Luc cells can be used as target and negative control cells respectively, to measure the induced anti-SV40 T-Ag immune response, but only after treatment with IFN-gamma.

Analysis of TAP-1 expression in HM-3. The HM-3 cells were also analyzed for expression of TAP-1 protein before and after treatment with IFN-gamma by Western blot analysis. As shown in Fig. 4.11, HM-3-Luc cells (lane 1) and HM-3-SV40-Luc cells (lane 3) do not show

detectable expression of TAP-1. However, upon treatment with IFN-gamma, both HM-3-Luc cells (lane 2) and HM-3-SV40-Luc (lane 4) show marked induction in the expression of TAP-1. Thus, similar to expression of MHC class I, the HM-3-Luc and HM-3-SV40-Luc cells, do not show detectable expression of TAP-1. However, like MHC class I expression again, the expression of TAP-1 was significantly induced upon treatment with IFN-gamma as shown by Western blot analysis. This confirms the results suggesting that HM-3-SV40-Luc and HM-3-Luc cells can be used as target and negative control cells respectively, to measure the induced anti-SV40 T-Ag immune response, but only after treatment with IFN-gamma.

Analysis of SV40 T-Ag expression in HM-3-SV40 tumors. Before using HM-3-SV40 cells as target cells to measure the induced immune response against SV40 T-Ag, it was important to confirm that the expression of SV40 T-Ag was retained in tumors formed *in vivo*. Thus, to determine expression of SV40 T-Ag, in HM-3-SV40 tumors, 5 X 10^7 HM-3-SV40 cells were injected i.p. into B6C3F1 mice. The mice were monitored for tumor growth; after approximately 4 weeks, the mice started to show signs of tumor growth and were sacrificed and examined histopathologically. The HM-3-SV40 cells formed both ascites as well as solid tumors *in vivo*. The cells from the ascites as well as solid tumors were cultured *in vitro* and tested for the expression of SV40 T-Ag. As shown in Figure 4.12, the tumor cells cultured from both, the ascites (lane 1) and solid tumor (lane 2) showed expression of SV40 T-Ag. Thus, HM-3-SV40 cells retain expression of the target tumor antigen, SV40 T-Ag, in tumors *in vivo*.

Survival studies and analysis of tumor growth in mice immunized with CD40-targeted Ad5-CMV-SV40 T-Ag. The gold standard for the evaluation of an anti-tumor vaccine is the survival studies of the immunized mice upon challenge with the tumor cells. The B6C3F1 mice were immunized with CD40-targeted as well as untargeted Ad5-CMV-SV40 T-Ag. Mice that received CD40-targeted Ad5-null, untargeted Ad5-null, CFm40L and PBS were used as controls. The immunized and control mice were challenged with IFN-γ treated IG10-SV40 tumor cells. A survival study was performed on these mice. Also, the tumor growth in these mice was analyzed by measuring change in their bodyweight every week.

As shown in figure 4.13, the mice immunized with CD40-targeted Ad5-CMV-SV40 T-Ag as well as untargeted Ad5-CMV-SV40 T-Ag showed 100% survival till 11 weeks after challenge with the tumor cells. While the control mice that received untargeted Ad5-null, CD40-targeted Ad5-null, CFm40L and PBS showed reduced survival rates of 50%, 65%, 78% and 57% respectively, at 11 weeks post-challenge. Likewise, the mice immunized with CD40-targeted and untargeted Ad5-CMV-SV40 T-Ag donot show change in their bodyweight, as significant as that shown by the control mice (figure 4.13). As shown in figure 4.13, at 11 weeks after challenge with the tumor cells, the mice immunized with CD40-targeted and untargeted Ad5-CMV-SV40 T-Ag showed an increase of only 20% in their body weight. While at 11 weeks, the mice that received only PBS showed the highest increase in their bodyweight, of approximately 86%. The animals that were immunized with untargeted Ad5-null, CD40-targeted Ad5-null and the CFm40L alone showed an increase in bodyweight of approximately 70%, 56% and 54%, respectively.

Thus, immunization with both CD40-targeted and untargeted Ad5-CMV-SV40 T-Ag confers protective immunity to B6C3F1 mice against tumor cells expressing SV40 T-Ag, leading to reduced tumor growth and enhanced survival of the tumor challenged mice. The immune response induced by immunization with CD40-targeted Ad5-CMV-SV40 T-Ag is similar to that induced by untargeted Ad5-CMV-SV40 T-Ag, as shown by their similar survival rates and changes in body weight upon challenge with tumor cells expressing SV40 T-Ag. Also, the anti-

tumor immune response induced is specific for SV40-T-Ag, as immunization with CD40-targeted and untargeted Ad5-null doesnot confer the same protective response against IG10-SV40 cells. Thus, CD40-targeted adenoviral vector expressing tumor antigen can successfully be used to generate antigen-specific prophylactic anti-tumor immunity in mice.

4.4 Discussion

The IG10 and HM-3 tumor cells were both successfully transfected with plasmids encoding SV40 T-Ag and luciferase as shown by the expression of SV40 T-Ag using Western blot analysis (Figs. 4.1 and 4.6) and expression of luciferase by bioluminescent imaging (Figs. 4.2, 4.7 and 4.8). SV40 T-Ag was the chosen target tumor antigen. Luciferase is an enzyme that acts on a substrate called luciferin and in the enzymatic reaction visible light is emitted. Introducing the *luciferase* gene in the tumor cells enables their visualization by bioluminescent imaging. The tumor forming potential of the stably transfected cells was confirmed in B6C3F1 mice injected with the stably transfected tumor cells.

The B6C3F1 mice injected with IG10-SV40 and IG10-Luc cells showed obvious signs of tumor growth at 4 weeks post-injection. The progression of tumor growth in the mice was also monitored by using bioluminescent imaging. As shown in Fig. 4.2, there was increase in the expression of luciferase in the B6C3F1 mice injected with IG10-Luc cells at 1 week post-injection compared with 1 day after injection, indicating proliferation of the IG10-Luc cells *in vivo* within the first week after injection. Thus, bioluminescent imaging can be used to follow the cell proliferation of IG10-Luc tumor cells in B6C3F1 mice. The IG10-SV40 and IG10-Luc cells formed both, ascites as well as solid tumors in B6C3F1 mice. The retention of SV40 T-Ag expression in tumors formed in mice injected with IG10-SV40 cells was confirmed by Western blot analysis as show in Fig. 4.5. The expression of the components of the antigen processing and presentation pathway such as MHC class I and TAP-1 was also determined to test the efficacy of the IG10-SV40 and IG10-Luc cells to serve as the target and control cells respectively. As shown in Figs. 4.3 and 4.4, both IG10-SV40 and IG10-Luc cells do not show significant expression of MHC class I and TAP-1 by themselves. However, both cell types show significant increase in the expression of both molecules on treatment with IFN-gamma. Thus, IG10-SV40 and IG10-Luc cells can be used as target tumor cells and control tumor cells respectively, but only on treatment with IFN-gamma.

The B6C3F1 mice injected with HM-3-Luc cells showed obvious tumor growth at 7 weeks post-injection. While the mice injected with HM-3-SV40-Luc cells showed obvious tumor growth at 4 weeks after injection. As shown in Fig. 4.7, there was significant increase in the expression of luciferase in B6C3F1 mice injected with HM-3-Luc cells at 7 weeks post-injection compared with 1 day after injection. While, as shown in Fig. 4.8, the mice injected with HM-3-SV40-Luc cells showed significant increase in the expression of luciferase at just 4 weeks after injection. Thus, with the demonstratio that bioluminescent imaging could be used to visualize and monitor the growth of HM-3-SV40-Luc and HM-3-Luc cells in B6C3F1 mice, these results also suggested the rate of growth of HM-3-SV40-Luc cells was greater than that of HM-3-Luc cells.

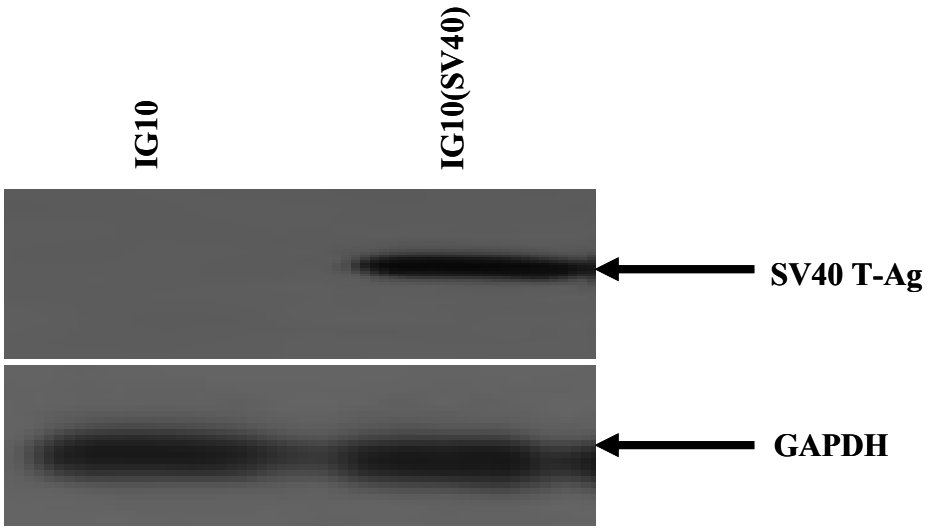
The *in vitro* analysis of the rate of growth of HM-3-SV40-Luc and HM-3-Luc cells confirmed the rate of proliferation of HM-3-SV40-Luc was greater than that of HM-3-Luc cells. This was not surprising as SV40 T-Ag is known to transform and confer proliferative advantage to cells when introduced into primary cells and established cell lines*[33, 34]*. The SV40 T-Ag disrupts cell growth control mechanisms primarily by binding to and inactivating the tumor

suppressor proteins p53 and pRB family proteins*[33]*. The fact that HM-3-SV40-Luc cells form tumors faster than HM-3-Luc cells in B6C3F1 mice was considered when doing the survival studies in B6C3F1 mice on immunization with CD40-targeted and untargeted Ad5-CMV-SV40 T-Ag.

Both HM-3-SV40-Luc and HM-3-Luc cells, can be used as the target tumor cells and control tumor cells in the survival studies, but only on treatment with inflammatory agents such as IFN-gamma. As shown in Figs. 4.9 and 4.10, the cells by themselves do not express significant MHC class I and TAP-1, however, there was significant induction in the expression of both of these molecules upon treatment with IFN-gamma. Both MHC class I and TAP-1 are essential for the efficient processing and presentation of tumor antigens on the cell-surface. Fig. 4.11 confirms the retention of expression of the target SV40 T-Ag in the tumors formed by HM-3-SV40-Luc cells in B6C3F1 mice. Thus, IFN-gamma treated HM-3-SV40-Luc cells and HM-3-Luc cells form a good pair of target tumor and control tumor cells respectively, to evaluate the anti-SV40 T-Ag immune response induced in B6C3F1 mice by CD40-targeted Ad5-CMV-SV40 T-Ag. In addition, the tumor progression of these cells can be followed *in vivo* by using bioluminescent imaging. However, the increase in bioluminescence was not consistent with increase in tumor growth at all time points and thus, changes in the weight of the animals should be monitored with bioluminescent imaging to follow the progression of tumor growth of these cell types in the B6C3F1 mice.

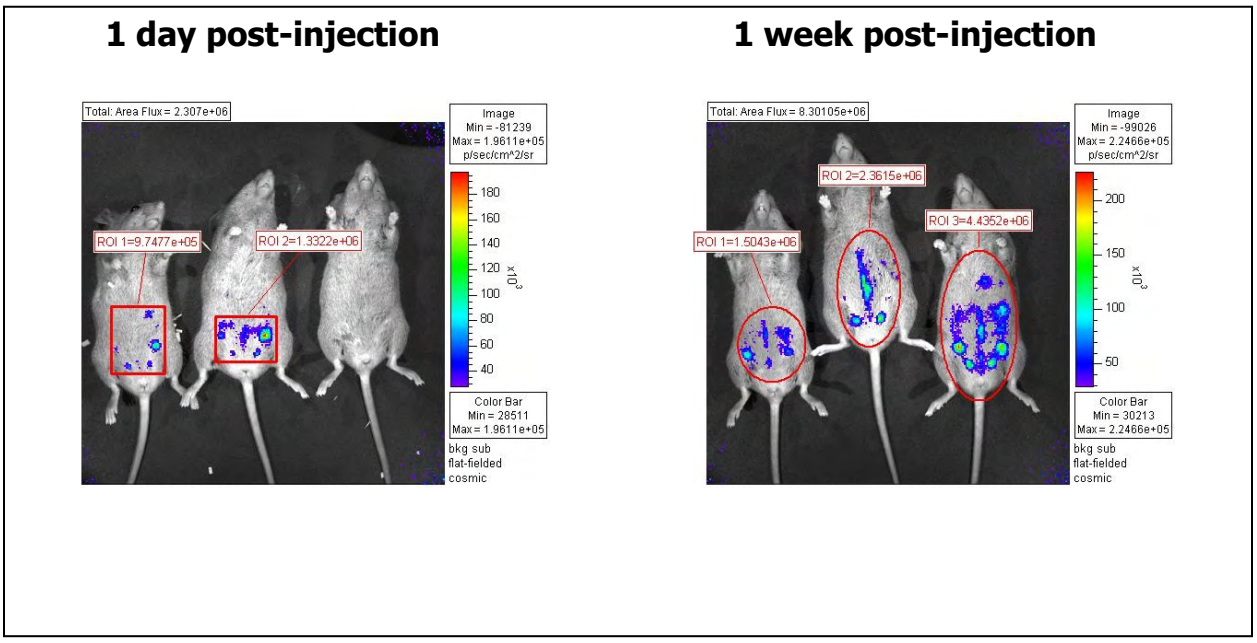
The IG10-SV40 tumor cells were used in the tumor challenge studies. The mice immunized with CD40-targeted Ad5-CMV-SV40 T-Ag and untargeted Ad5-CMV-SV40 T-Ag both showed increased survival and reduced tumor growth compared to the control mice. The increase in survival and reduction in tumor growth elicited by CD40-targeted Ad5-CMV-SV40 T-Ag was comparable to that induced by untargeted Ad5-CMV-SV40 T-Ag. Thus, the prophylactic anti-tumor immune response induced by CD40-targeted Ad5-CMV-SV40 T-Ag is similar to that generated by untargeted Ad5-CMV-SV40 T-Ag.

Figure 4.1
IG10 cells stably transfected with an
SV40 T-Ag expression plasmid



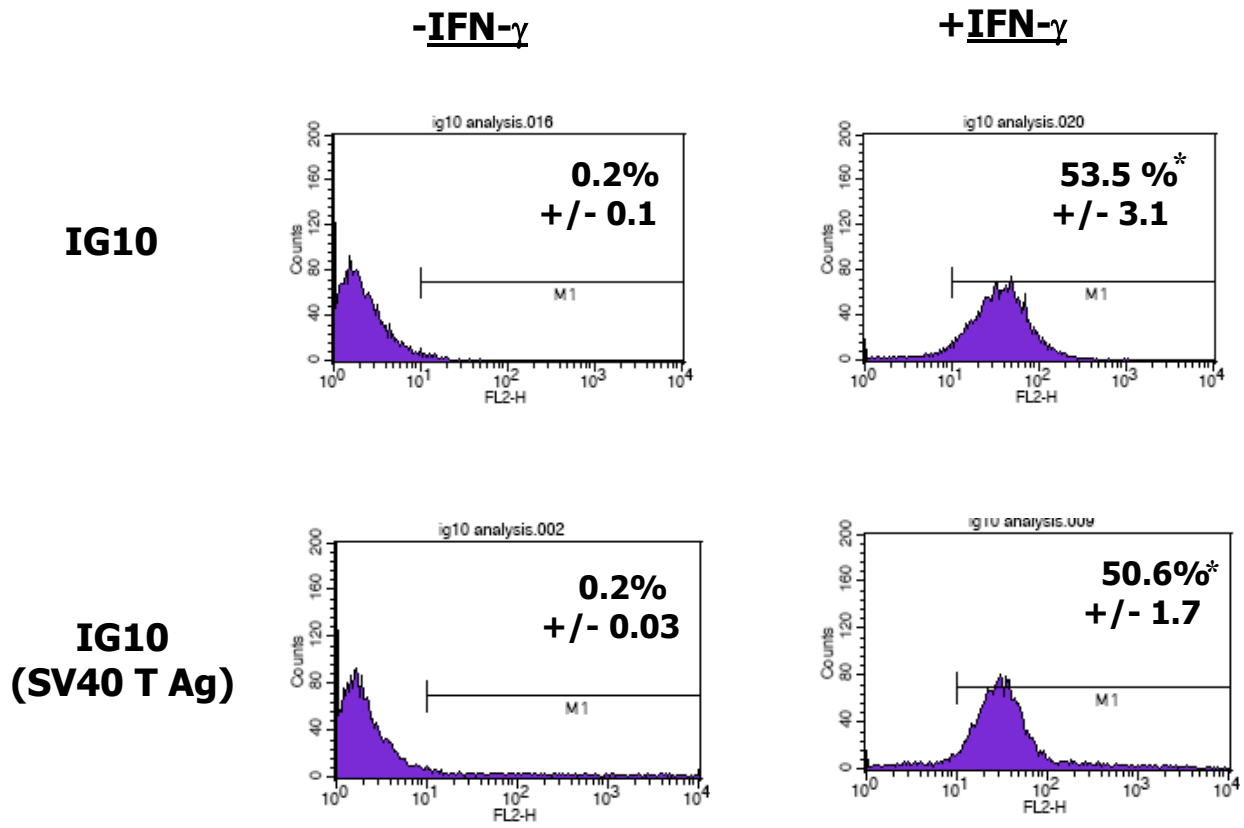
Western blot assay of IG10-Luc and IG10-SV40 cells. The expression of SV40 T-Ag was detected in IG10-SV40-Luc cells (lane 2) but not in IG10-Luc cells (lane 1).

Figure 4.2
Tumorigenicity of IG10 Cells



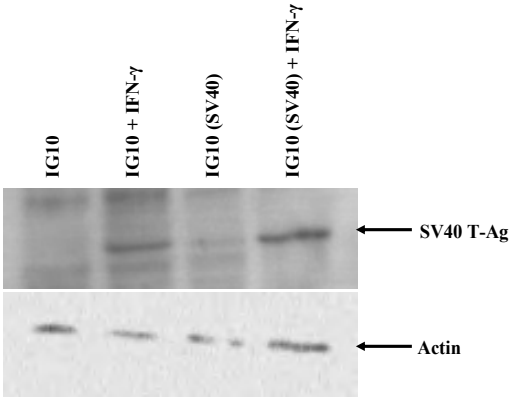
Bioluminescent Imaging. B6C3F1 mice were injected with IG10-Luc cells. Three mice were injected with 1 X 10^7 cells per mouse. The mice were subjected to bioluminescent imaging at 1 day and then 1 week after injection. The signal bars indicate relative photons light intensity.

Figure 4.3
IG10 cells show an increase in expression of MHC-I after treatment with IFN-γ



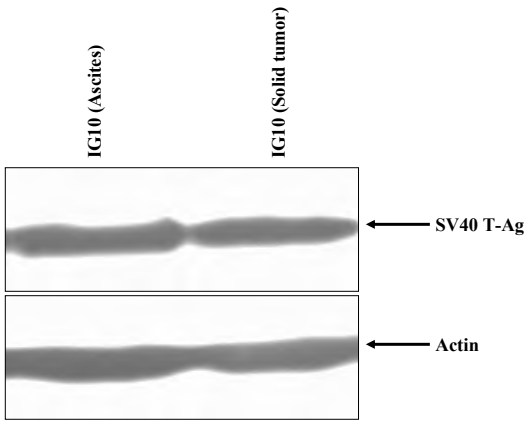
Flow cytometric analysis of the IG10-Luc and IG10-SV40-Luc cells without and with IFN-gamma treatment. The cells were stained with FITC-labeled antibody against MHC class I.
* P < 0.05 versus control IG10 and IG10(SV40) cells, not treated with IFN-g.

Figure 4.4
IG10 cell line shows an increase in expression of TAP-1 after treatment with IFN-γ



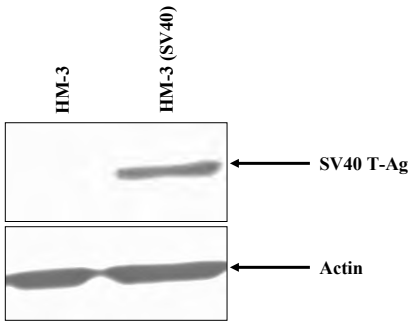
Western blot assay of IG10 and IG10-SV40 cells before and after treatment with IFN-gamma. The expression of TAP-1 was detected in IG10 and IG10-SV40 cells only after treatment with IFN-gamma. The IFN-gamma treated IG10 cells (lane 2) and IG10-SV40 (lane 4) cells show expression of TAP-1, but not the untreated IG10 and IG10-SV40 cells (lanes 1 and 3).

Figure 4.5
IG10 cells cultured from solid tumor and ascites show expression of SV40 T-Ag



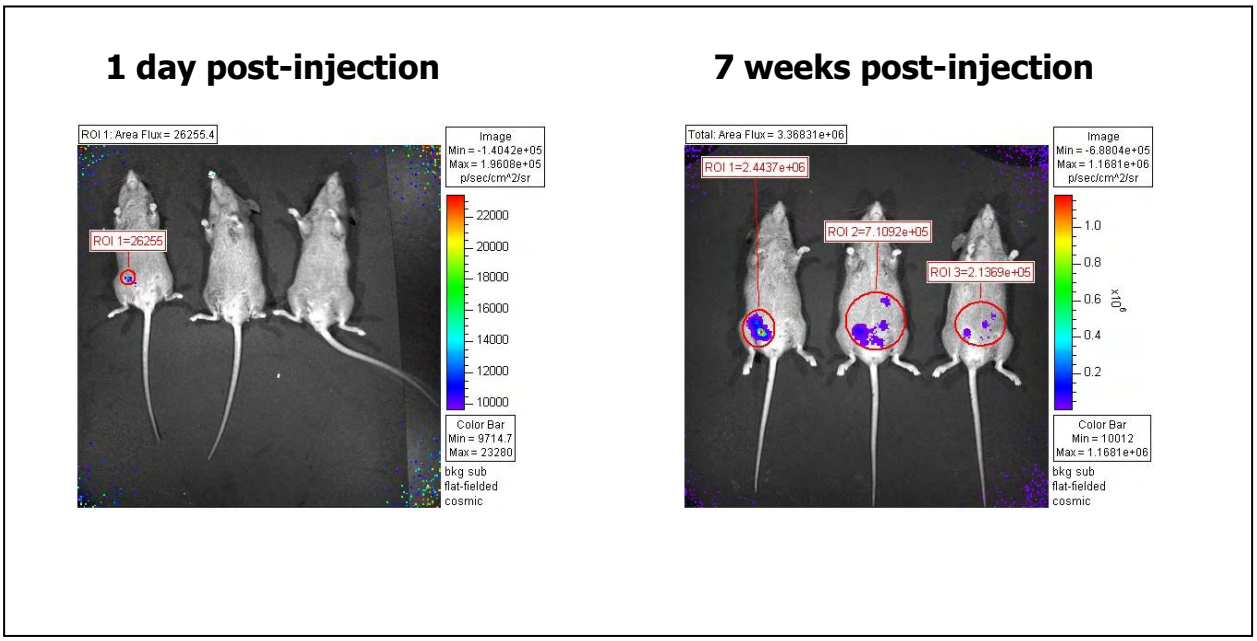
Western blot assay of IG10-SV40 cells. The expression of SV40 T-Ag was detected in tumor cells cultured from the ascites (lane 1) and cells cultured from the solid tumor (lane 2).

Figure 4.6
HM-3 cells stably transfected
with an SV40 T-Ag expression plasmid



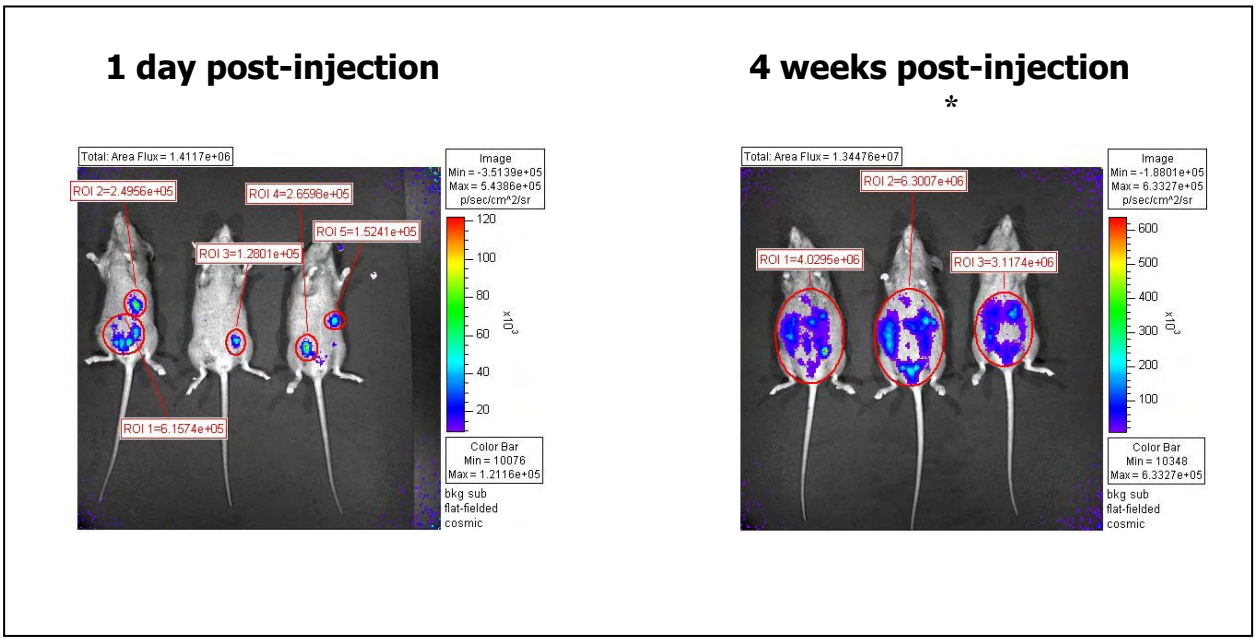
Western blot assay of HM-3-Luc and HM-3-SV40-Luc cells. The expression of SV40 T-Ag was detected in HM-3-SV40-Luc cells (lane 2) but not in HM-3-Luc cells (lane 1).

Figure 4.7
Tumorigenicity of HM-3 cells



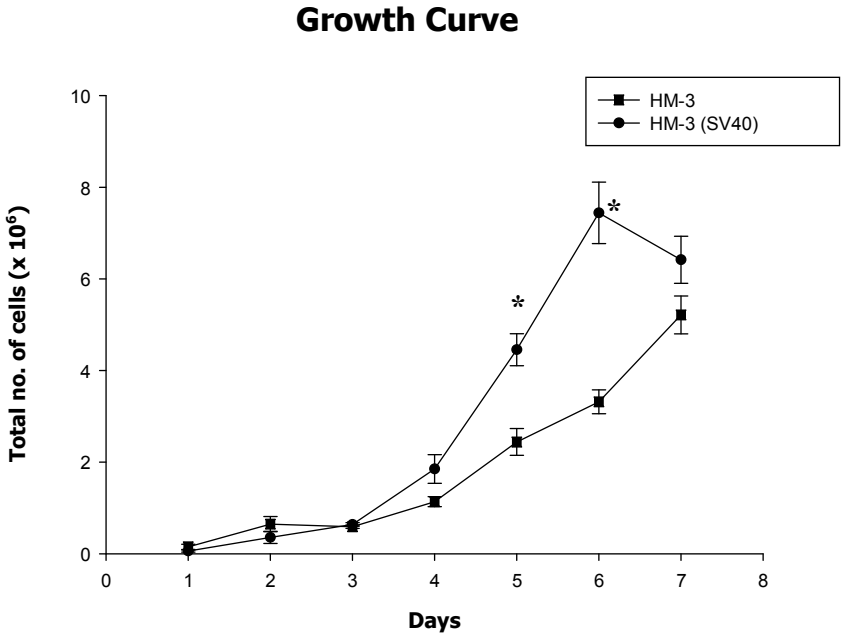
Bioluminescent Imaging. B6C3F1 mice were injected with HM-3-Luc cells. Three mice were injected with 1 X 10^7 cells per mouse. The mice were subjected to bioluminescent imaging at 1 day and then every week after injection until 7 weeks. The signal bars indicate relative photons light intensity.

Figure 4.8
Tumoriginicity of HM-3 (SV40) cells



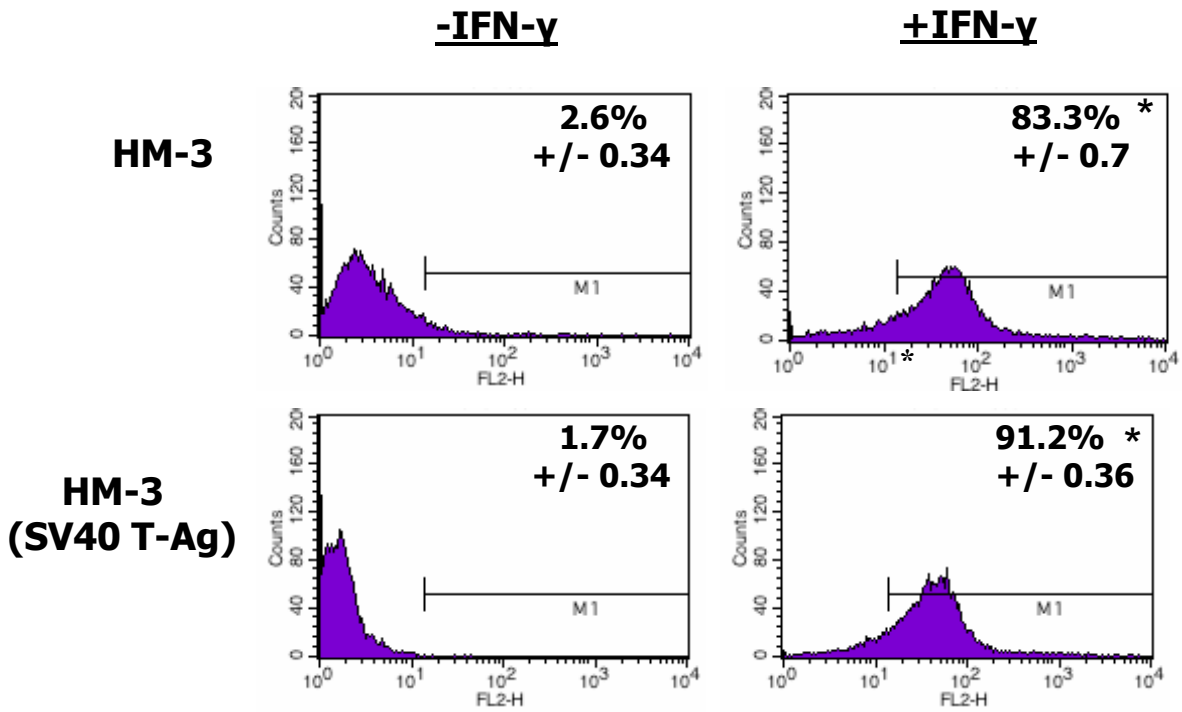
Bioluminescent Imaging. B6C3F1 mice were injected with HM-3-SV40-Luc cells. Three mice were injected with 1 X 10^7 cells per mouse. The mice were subjected to bioluminescent imaging at 1 day and then every week after injection until 4 weeks. The signal bars indicate relative photons light intensity. * $P < 0.05$ versus mice injected with HM-3(SV40) cells at 1 day post-injection.

Figure 4.9
HM-3 (SV40) cells have a higher growth curve than HM-3 cells



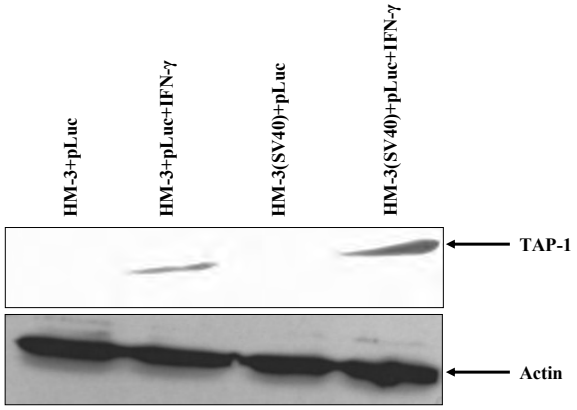
Growth curves of HM-3 and HM-3(SV40) cells. Each cell group was maintained in DMEM media supplemented with 10% FBS and cells numbers were counted on the indicated days. Mean no. of cells +/- SE obtained from three observations, is plotted against the number of

Figure 4.10
HM-3 Cells Show an Increase in Expression of MHC-I after Treatment with IFN-γ



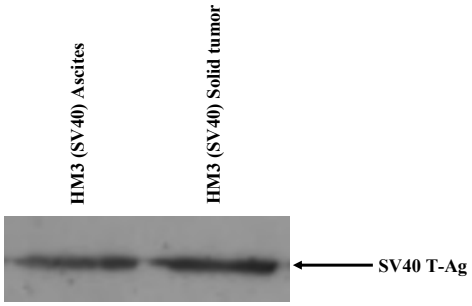
Flow cytometric analysis of the HM-3-Luc and HM-3(SV40)-Luc cells without and with IFN-gamma treatment. The cells were stained with FITC-labeled antibody against MHC class I. * $P < 0.05$ versus control HM-3 and HM-3(SV40) cells, not treated with IFN-g.

Figure 4.11
HM-3 Cell Line Shows an Increase in Expression of TAP-1 after Treatment with IFN-γ



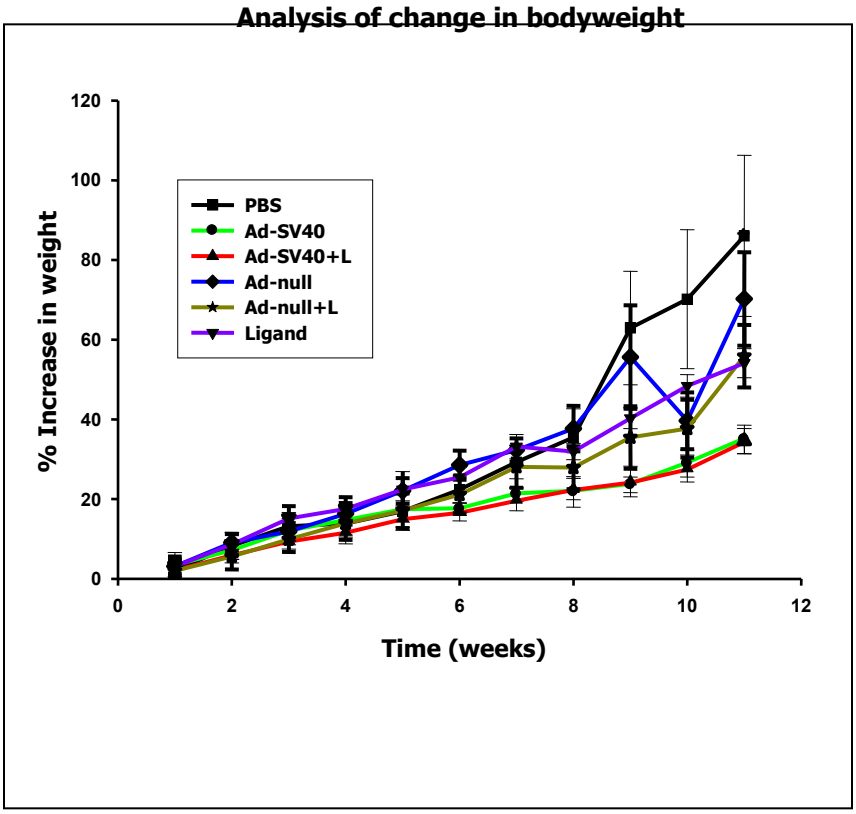
Western blot assay of HM-3 and HM-3-SV40 cells before and after treatment with IFN-gamma. The expression of TAP-1 was detected in HM-3 and HM-3-SV40 cells only after treatment with IFN-gamma. The IFN-gamma treated HM-3 cells (lane 2) and HM-3-SV40 (lane 4) cells show expression of TAP-1, but not the untreated HM-3 and HM-3-SV40 cells (lanes 1 and 3).

Figure 4.12
HM-3 Cells Cultured from Solid Tumor and Ascites Show Expression of SV40 T-Ag



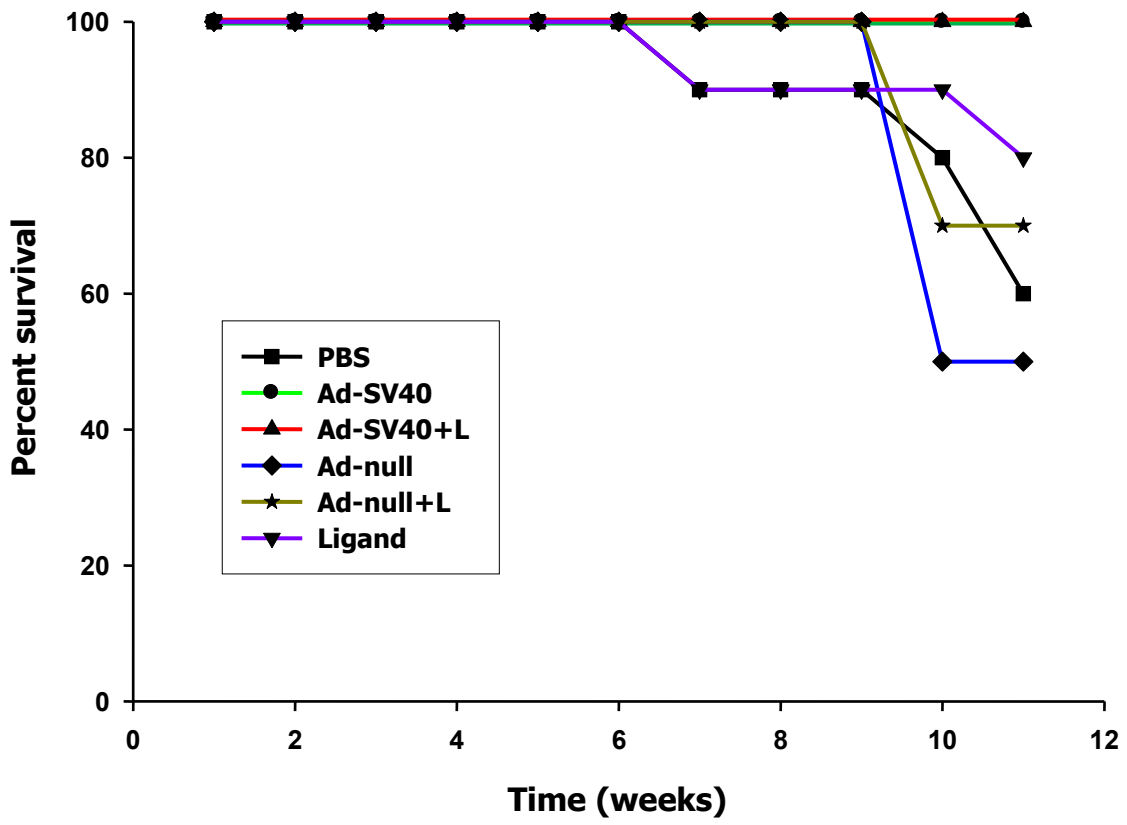
Western blot assay of HM-3-SV40 cells. The expression of SV40 T-Ag was detected in tumor cells cultured from the ascites (lane 1) and cells cultured from the solid tumor (lane 2).

Figure 4.13
Tumor Challenge studies



Analysis of percentage change in bodyweight . The B6C3F1 mice immunized with CD40-targeted Ad5-CMV-SV40 T-Ag (Ad-SV40+L) and untargeted Ad5-CMV-SV40 T-Ag (Ad-SV40) were challenged with IG10-SV40 tumor cells. The mice immunized with CD40-targeted Ad5-null (Ad-null+L), untargeted Ad5-null (Ad5-null), CFm40L (Ligand) and saline (PBS), also challenged with IG10-SV40 tumor cells, were used as controls. The mice immunized with CD40-targeted and untargeted Ad5-CMV-SV40 T-Ag showed similar percentage of increase in bodyweight, which was significantly less ($P < 0.05$) than that shown by the control mice. * $P < 0.05$ versus mice immunized with CD40-targeted Ad5-null (Ad-null+L), untargeted Ad5-null (Ad-null), CFm40L (Ligand) and saline (PBS).

Figure 4.14
Tumor Challenge Studies



Analysis of survival . The B6C3F1 mice immunized with CD40-targeted Ad5-CMV-SV40 T-Ag (Ad-SV40+L) and untargeted Ad5-CMV-SV40 T-Ag (Ad-SV40) were challenged with IG10-SV40 tumor cells. The mice immunized with CD40-targeted Ad5-null (Ad-null+L), untargeted Ad5-null (Ad5-null), CFm40L (Ligand) and saline (PBS), also challenged with IG10-SV40 tumor cells, were used as controls. The mice immunized with CD40-targeted and untargeted Ad5-CMV-SV40 T-Ag showed 100% survival until 11 weeks after challenge with the tumor cells, while the control mice showed significant decrease in their survival.

5. SUMMARY AND CONCLUSIONS

Our results show that CD40-targeted Ad5-CMV-SV40 T-Ag successfully elicits a protective SV40 T-Ag specific anti-tumor immune response in mice and induces reduced liver toxicity compared to untargeted Ad5-CMV-SV40 T-Ag. To the best of our knowledge, this is the first study to show a modified Ad5 vector to be both, potent and safe. Several studies have demonstrated either potency or reduced liver toxicity of modified adenoviral vectors, but not both. Curiel et al have shown tumor targeting and hepatic untargeting by a Coxackie/Adenovirus receptor (sCAR) ectodomain anti-carcinoembryonic antigen (MFE) bispecific adaptor*[25]*. However, our results have not only shown successful DC-targeting and hepatic untargeting, but also successful induction of anti-tumor immune response and reduced liver inflammation by CD40-targeted adenovirus compared to untargeted adenovirus. According to our knowledge, our study is the first to demonstrate potency as well as safety of a therapeutic targeted adenoviral vector *in vivo*.

The goal was to target antigen presenting cells like DCs using adenoviral vector and express tumor antigens in them to elicit an anti-tumor immune response. However, adenovirus cannot efficiently infect DCs because of deficiency of CAR on DC cell surface. Thus, we used a bispecific adapter molecule called CFm40L, to target adenovirus to cells that express CD40, which includes DCs. CD40-targeted adenoviral vectors are known to infect DCs and induce antigen specific anti-tumor immune response more efficiently than untargeted adenovirus*[5]*. Our results also showed that DCs were more efficiently infected by CD40-targeted Ad5-CMV-SV40 T-Ag than untargeted Ad5-CMV-SV40 T-Ag. The DCs infected by CD40-targeted Ad5-CMV-SV40 T-Ag showed increased potential for activating lymphocytes that was marked by elevated phenotypic expression of co-stimulatory molecules and enhanced secretion of inflammatory cytokines and chemokines by infected DCs. Thus, our results demonstrated that CD40-targeted Ad5-CMV-SV40 T-Ag can efficiently infect APCs *in vitro* and increase their activation potential by increasing their maturation status.

Our results also showed an antigen-specific CTL response and a prophylactic anti-tumor immune response in B6C3F1 mice immunized with CD40-targeted Ad5-CMV-SV40 T-Ag. The results from both, the *in vivo* CTL assay and survival studies of the B6C3F1 mice showed that the anti-SV40 T-Ag immune response induced by CD40-targeted Ad5-CMV-SV40 T-Ag was almost equivalent to that induced by untargeted Ad5-CMV-SV40 T-Ag. This was not in accordance with our hypothesis that CD40-targeted Ad5-CMV-SV40 T-Ag would infect DCs more efficiently than untargeted Ad5-CMV-SV40 T-Ag, and will thus induce a greater SV40 T-Ag specific anti-tumor immune response in mice than untargeted Ad5-CMV-SV40 T-Ag. One possibility is that SV40 T-Ag is a highly immunogenic antigen and thus even minimal infection that may be achieved by untargeted Ad5-CMV-SV40 T-Ag is sufficient to induce an immune response as strong as that induced by CD40-targeted Ad5-CMV-SV40 T-Ag. And thus, there was no difference observed in the killing of target cells by CTLs in the *in vivo* CTL assay, and in the survival of tumor-challenged B6C3F1 mice immunized by CD40-targeted and untargeted Ad5-CMV-SV40 T-Ag. Another possibility is that though there was no difference observed in the immunization potential of CD40-targeted and untargeted Ad5-CMV-SV40 T-Ag in the prophylactic model, the therapeutic model could reveal the probable difference in between the immune responses. It is much more difficult to achieve therapeutic immunity than it is to achieve protective immunity by anti-cancer vaccines because the tumor cells have already evolved and adopted various strategies to avoid elimination by the immune system, such as

down-modulation of MHC expression, induction of regulatory T-cells, *etc.* Therefore, though there was no difference in the protective immunity induced by CD40-targeted and untargeted Ad5-CMV-SV40 T-Ag, it cannot be ruled out there might be a difference in their potential to induce therapeutic immunity. A third possibility is that although CD40-targeted Ad5-CMV-SV40 T-Ag generated an anti-SV40 T-Ag specific immune response similar to that induced by untargeted Ad5-CMV-SV40 T-Ag in naïve B6C3F1 mice, it may generate an enhanced immune response compared to the untargeted Ad5-CMV-SV40 T-Ag in mice pre-exposed to adenovirus. Mice pre-exposed to adenovirus develop neutralizing antibodies to adenovirus, which dampen the efficacy of the adenoviral gene therapy. Prior exposure to adenovirus has primed most humans with rapid neutralizing antibody against re-exposure to adenovirus.

The presence of Ad5-specific neutralizing antibody is one of the major obstacles in the use Ad5 serotype for systemic gene therapy. Various approaches used to overcome this obstacle include: 1) deletion of viral genes to reduce the number of viral proteins synthesized*[19-21]*, *e.g.,* gutless adenovirus, and 2) modification of the capsid proteins, genetic as well as non-genetic, such that they are less immunogenic*[22]*. The CD40-targeted adenovirus is coated with the CFm40L bi-specific adaptor molecule and thus the antigenic fiber knob domain of the virus and other cell surface structures are masked. As a result, CD40-targeted adenovirus may be able to sustain longer *in vivo* than untargeted adenovirus in the presence of Ad-specific neutralizing antibodies, and thus have a higher therapeutic index. Studies by Huang et al, which show that neutralizing antibodies had no inhibitory effects on CFm40L mediated Ad-luc transduction of human DCs *in vitro*, supports this possibility*[35]*. Regardless of all possibilities, there is no doubt that the CD40-targeted Ad5-CMV-SV40 T-Ag is an effective adenoviral vaccine, whose potency is as good as that of untargeted Ad5-CMV-SV40 T-Ag, if not better.

We also observed reduced liver toxicity in B6C3F1 mice immunized with CD40-targeted Ad5-CMV-SV40 T-Ag compared with mice immunized with untargeted Ad5-CMV-SV40 T-Ag. Our hypothesis was that by targeting adenovirus to CD40, the natural hepato-tropism of the adenovirus is altered, leading to hepatic un-targeting and reduced liver toxicity. Our results showed there was reduced infection and inflammation of the liver in B6C3F1 mice immunized with CD40-targeted Ad5-CMV-SV40 T-Ag than those immunized with untargeted Ad5-CMV-SV40 T-Ag. Thus, our results support our hypothesis that targeting adenovirus to CD40, de-targets it from the liver, resulting in reduced liver inflammation compared with untargeted adenovirus. Therefore, our studies demonstrate that CD40-targeted Ad5-CMV-SV40 T-Ag is a safer vaccine than untargeted Ad5-CMV-SV40 T-Ag.

The generation of an effective anti-tumor immunity consists of two components: a) successful induction of anti-tumor immune response, and b) effective killing of the tumor cells mediated by activated immune cells, *i.e.,* a successful effector phase. Defects in the induction of the immune response due to poor-presentation of the tumor antigens by tumor cells, can be overcome with CD40-targeted adenoviral vector as it targets expression of tumor antigens to professional antigen presenting cells like the DCs. However, other factors or defects that render the tumor cells invisible to the immune system or resistant to killing by the immune cells, such as expression of poorly immunogenic antigens, down-modulation or loss of expression of MHC by tumor cells, secretion of immunosuppressive cytokines or induction of regulatory T-cells, etc. cannot be overcome by using the CD40-targeted adenoviral vaccine by itself*[9]*. In that situation other adjuvants, like cytokine therapy to increase the inflammation in the tumor milieu, neutralizing antibodies for immunosuppressive cytokines such as IL-10 and TGF-β or depletion of regulatory T-cells along with induction of immune response using CD40-targeted adenoviral

vaccine will be necessary for the successful elimination of tumor cells. Another possible approach to overcome the above problems would be to use highly immunogenic antigens not expressed by the tumor cells to immunize the diseased animals using CD40-targeted adenoviral vaccine, and then use tumor targeted vectors to express the immunogenic antigen specifically in the tumor cells. This would mimic the prophylactic model of immunization where the antigen-specific immune response is already induced and active before the antigen is expressed in the tumor cells and thus the infected tumor cells will be more sensitive to killing by the activated immune cells. This approach would have several advantages such as exploitation of known immunogenic antigens to induce a strong antigen specific immune response, expression of the antigens specifically by the tumor cells, thus avoiding the possibility of an autoimmune response. This strategy would also help to overcome the strategies used by the tumor cells to avoid immune-mediated killing, such as down-modulation of expression of the tumor specific antigen, induction of antigen specific regulatory T-cells, *etc.* The CTLs and other immune cells which are already activated, will detect and kill the tumor cells expressing the antigen, before they have had a chance to evolve and adopt these strategies, taking advantage of the prophylactic mode of immunization.

Apart from its immunization potential, CD40-targeted adenoviral vector can also be used in evaluating immunogenicity of newly identified tumor antigens. The major challenge in making efficient vaccines against tumor antigens is identifying immunogenic antigens against which an effective immune response can be induced. Thus, selecting highly immunogenic antigens will greatly increase the prospects of making an effective anti-tumor vaccine. The current process of evaluating the target antigens for their potential use in vaccination is time-consuming, cumbersome and less efficient*[36]*. CD40-targeted adenoviral vectors expressing target antigen can be used to infect APCs *in vivo* and induce an antigen-specific immune response. The induced immune responses can then be measured and compared with compare the immunogenicity of different target antigens. This method has the advantage of using the APC machinery to address questions about antigen processing and HLA binding, rather than trying to reconstitute antigen processing *in vitro*, predicting and then testing the binding of peptides to MHC molecules *in vitro*. In addition, this is an *in vivo* model, which is less likely to be influenced by external factors.

Thus, the ability of CD40-targeted adenovirus expressing target antigen to induce protective antigen-specific immunity *in vivo* without causing significant liver toxicity makes it an attractive candidate for use as anti-tumor vaccine. Future studies involve pre-clinical evaluation of CD40-targeted Ad vectors to induce specific therapeutic immunity using clinically relevant ovarian tumor antigens, such as MUC-1.

KEY RESEARCH ACCOMPLISHMENTS

1 CD40-Targeted Adenoviral Vector Transduction of Dendritic Cells (2007) Disha A. Mody, Yoshinobu Odaka, Jagat Podduturi, Xiao L. Li, David T. Curiel, Alexander V. Pereboev, and J. Michael Mathis. European Society of Cell and Gene Therapy Fifteenth Annual Meeting. October 27 - 30. Abstract P257.

2 CD40-Targeted Adenoviral Vector Transduction of Dendritic Cells (2007) Disha Mody, Yoshinobu Odaka, Jagat Poduturri, Xiao L. Li, David T. Curiel, Alexander V. Pereboev, and J. Michael Mathis. American Society of Gene Therapy Tenth Annual Meeting. May 30 - June 3. Abstract 33.

REPORTABLE OUTCOMES

Task 3

- We showed that IFN-gamma treated HM-3-SV40-Luc cells and HM-3-Luc cells form a good pair of target tumor and control tumor cells respectively, to evaluate the anti-SV40 T-Ag immune response induced in B6C3F1 mice by CD40-targeted Ad5-CMV-SV40 T-Ag. In addition, the tumor progression of these cells can be followed *in vivo* by using bioluminescent imaging. However, the increase in bioluminescence was not consistent with increase in tumor growth at all time points and thus, changes in the weight of the animals should be monitored with bioluminescent imaging to follow the progression of tumor growth of these cell types in the B6C3F1 mice.
- We found that the prophylactic anti-tumor immune response induced by CD40-targeted Ad5-CMV-SV40 T-Ag is similar to that generated by untargeted Ad5-CMV-SV40 T-Ag.
- The IG10 and HM-3 tumor cells were both successfully transfected with plasmids encoding SV40 T-Ag and luciferase as shown by the expression of SV40 T-Ag using Western blot analysis and expression of luciferase by bioluminescent imaging.
- The B6C3F1 mice injected with IG10-SV40 and IG10-Luc cells showed obvious signs of tumor growth at 4 weeks post-injection. The progression of tumor growth in the mice was also monitored by using bioluminescent imaging. There was increase in the expression of luciferase in the B6C3F1 mice injected with IG10-Luc cells at 1 week post-injection compared with 1 day after injection, indicating proliferation of the IG10-Luc cells *in vivo* within the first week after injection.
- We confirmed the retention of SV40 T-Ag expression in tumors formed in mice injected with IG10-SV40 cells y Western blot analysis. The expression of the components of the antigen processing and presentation pathway such as MHC class I and TAP-1 was also determined to test the efficacy of the IG10-SV40 and IG10-Luc cells to serve as the target and control cells respectively. Both IG10-SV40 and IG10-Luc cells do not show significant expression of MHC class I and TAP-1 by themselves. However, both cell types show significant increase in the expression of both molecules on treatment with IFN-gamma.
- The B6C3F1 mice injected with HM-3-Luc cells showed obvious tumor growth at 7 weeks post-injection. While the mice injected with HM-3-SV40-Luc cells showed obvious tumor growth at 4 weeks after injection. As shown in Fig. 4.7, there was significant increase in the expression of luciferase in B6C3F1 mice injected with HM-3-Luc cells at 7 weeks post-injection compared with 1 day after injection. While, as shown in Fig. 4.8, the mice injected

with HM-3-SV40-Luc cells showed significant increase in the expression of luciferase at just 4 weeks after injection.

- We confirmed the *in vitro* analysis of the rate of growth of HM-3-SV40-Luc and HM-3-Luc cells confirmed the rate of proliferation of HM-3-SV40-Luc was greater than that of HM-3-Luc cells. This was not surprising as SV40 T-Ag is known to transform and confer proliferative advantage to cells when introduced into primary cells and established cell lines. The SV40 T-Ag disrupts cell growth control mechanisms primarily by binding to and inactivating the tumor suppressor proteins p53 and pRB family proteins*[33]*. The fact that HM-3-SV40-Luc cells form tumors faster than HM-3-Luc cells in B6C3F1 mice was considered when doing the survival studies in B6C3F1 mice on immunization with CD40-targeted and untargeted Ad5-CMV-SV40 T-Ag.
- We demonstrated that both HM-3-SV40-Luc and HM-3-Luc cells, can be used as the target tumor cells and control tumor cells in the survival studies, but only on treatment with inflammatory agents such as IFN-gamma. The cells by themselves do not express significant MHC class I and TAP-1, however, there was significant induction in the expression of both of these molecules upon treatment with IFN-gamma. Both MHC class I and TAP-1 are essential for the efficient processing and presentation of tumor antigens on the cell-surface.
- We confirmed the retention of expression of the target SV40 T-Ag in the tumors formed by HM-3-SV40-Luc cells in B6C3F1 mice.
- We used the IG10-SV40 tumor cells in tumor challenge studies. The mice immunized with CD40-targeted Ad5-CMV-SV40 T-Ag and untargeted Ad5-CMV-SV40 T-Ag both showed increased survival and reduced tumor growth compared to the control mice. The increase in survival and reduction in tumor growth elicited by CD40-targeted Ad5-CMV-SV40 T-Ag was comparable to that induced by untargeted Ad5-CMV-SV40 T-Ag.
- The results from the survival studies of the B6C3F1 mice showed that the tumor protective effect induced by CD40-targeted Ad5-CMV-SV40 T-Ag was almost equivalent to that induced by untargeted Ad5-CMV-SV40 T-Ag.

CONCLUSIONS

- Bioluminescent imaging can be used to follow the cell proliferation of IG10-Luc tumor cells in B6C3F1 mice. The IG10-SV40 and IG10-Luc cells formed both, ascites as well as solid tumors in mice
- IG10-SV40 and IG10-Luc cells can be used as target tumor cells and control tumor cells respectively, but only on treatment with IFN-gamma. B6C3F1 mice.
- With the demonstratio that bioluminescent imaging could be used to visualize and monitor the growth of HM-3-SV40-Luc and HM-3-Luc cells in B6C3F1 mice, these results also suggested the rate of growth of HM-3-SV40-Luc cells was greater than that of HM-3-Luc cells.
- The results from the survival studies was not in accordance with our hypothesis that CD40-targeted Ad5-CMV-SV40 T-Ag would infect DCs more efficiently than untargeted Ad5-CMV-SV40 T-Ag, and will thus induce a greater SV40 T-Ag specific anti-tumor immune response in mice than untargeted Ad5-CMV-SV40 T-Ag. One possibility is that SV40 T-Ag

is a highly immunogenic antigen and thus even minimal infection that may be achieved by untargeted Ad5-CMV-SV40 T-Ag is sufficient to induce an immune response as strong as that induced by CD40-targeted Ad5-CMV-SV40 T-Ag. Another possibility is that though there was no difference observed in the immunization potential of CD40-targeted and untargeted Ad5-CMV-SV40 T-Ag in the prophylactic tumor model, a therapeutic model could reveal a difference in between the immune responses. It is much more difficult to achieve therapeutic immunity than it is to achieve protective immunity by anti-cancer vaccines because the tumor cells have already evolved and adopted various strategies to avoid elimination by the immune system, such as down-modulation of MHC expression, induction of regulatory T-cells, *etc.* A third possibility is that although CD40-targeted Ad5-CMV-SV40 T-Ag generated a the tumor protective effect similar to that induced by untargeted Ad5-CMV-SV40 T-Ag in naïve B6C3F1 mice, it may generate an enhanced immune response compared to the untargeted Ad5-CMV-SV40 T-Ag in mice pre-exposed to adenovirus. Mice pre-exposed to adenovirus develop neutralizing antibodies to adenovirus, which dampen the efficacy of the adenoviral gene therapy. Prior exposure to adenovirus has primed most humans with rapid neutralizing antibody against re-exposure to adenovirus.

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Abstract Title:

CD40-Targeted Adenoviral Vector Transduction of Dendritic Cells

Abstract Text:

Dendritic cells (DCs) are professional antigen presenting cells of the immune system. Targeting DCs with tumor antigens can result in DC-mediated presentation of the tumor antigen to the immune system and elicit tumor-specific immune response. DCs are difficult to transduce by using an adenovirus (Ad) vector because of the scarcity of CAR on the DC cell surface. CD40 is a cell surface marker expressed by DCs, is crucial for their maturation and the subsequent activation of the immune system by the DCs. We explored the possibility of targeting DCs using Ad vector via CD40. We used CFm40L, a bispecific adaptor molecule with the ectodomain of CAR linked by a trimerization motif to the extracellular domain of mouse CD40 ligand. CFm40L bridges the fiber knob domain of Ad to CD40 on mouse DC. The SV40 T-Ag is known to be a highly immunogenic protein. We used SV40 T-Ag as a model antigen to test the efficacy of CD40-targeted Ad vaccine to tranduce DCs and generate an effective immune response against tumor cells bearing SV40 T-Ag. Western blot analysis was used to detect expression of SV40 T-Ag in the DCs transduced with CD40-targeted and untargeted Ad-SV40 T-Ag. Chromium release assays were used to test the Cytotoxic T Lymphocyte (CTL) response generated in the immunized mice against cells bearing SV40 T-Ag. Morphological and histological examination of the liver was performed to compare the liver toxicity induced in mice by CD40-targeted Ad-SV40 TAg and untargeted Ad-SV40 T-Ag. Real-time PCR analysis of Ad DNA in the liver samples was used to assess targeting of liver in mice by the untargeted and CD40-targeted Ad5. MicroPET scans were performed to measure the expression of a transgene TK (thymine kinase) in the livers of mice immunized with CD40-targeted and untargeted Ad expressing TK. DCs transduced by using CD40-targeted Ad-SV40 T-Ag showed increased expression of SV40 T-Ag. The mice immunized with CD40-targeted Ad-SV40 T-Ag showed a

greater CTL response than mice immunized with untargeted Ad-SV40 T-Ag, and there was reduced liver toxicity. CD40-targeted Ad vectors showed lower transduction of liver and expression of TK. These results demontrsated that CD40-targeted Ad vaccine is a more potent and safer vaccine than untargeted Ad vaccine.

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Title: CD40-Targeted Adenoviral Vector Transduction of Dendritic Cells
Disha Mody[1], Don A. Sibley, Ph.D.[1], Yoshinobu Odaka[1], Jagat Poduturri[1], Xiao L. Li[1], David T. Curiel, M.D., Ph.D.[2], Alexander Pereboev, Ph.D.[2] and J. Michael Mathis, Ph.D.[1]. [1]Cellular Biology and Anatomy, Gene Therapy Program, LSU Health Sciences Center, Shreveport, LA, United States, 71130 and [2]Division of Human Gene Therapy, Departments of Medicine, Obstetrics and Gynecology, Pathology, and Surgery, and the Gene Therapy Center, University of Alabama at Birmingham, Birmingham, AL, United States, 35294.
Body:
Introduction: Dendritic cells (DCs) are professional antigen presenting cells of the immune system. Targeting DCs with tumor antigens can result in DC-mediated presentation of the tumor

antigen to the immune system and elicit tumor-specific immune response. DCs are difficult to transduce by using an adenovirus (Ad) vector because of the scarcity of CAR on the DC cell surface. CD40 is a cell surface marker expressed by DCs, is crucial for their maturation and the subsequent activation of the immune system by the DCs. We have explored the possibility of targeting DCs using Ad vector via CD40. We have used CFm40L, a bispecific adaptor molecule with ectodomain of CAR one end end linked genetically by a trimerization motif to the extracellular domain of mouse CD40 ligand on the other end. CFm40L bridges the fiber knob domain of Ad to CD40 on mouse DC. The SV40 T-Ag is known to be a highly immunogenic protein. We used SV40 T-Ag as a model antigen to test the efficacy of CD40-targeted Ad vaccine to tranduce DCs and generate an effective immune response against tumor cells bearing SV40 T-Ag.

Methods: Western blot analysis was used to detect expression of SV40 T-Ag in the DCs transduced with CD40-targeted and untargeted Ad-SV40 T-Ag. Chromium release assays were used to test the Cytotoxic T Lymphocyte response generated in the immunized B6C3F1 mice against cells bearing SV40 T-Ag. Morphological and histological examination of the liver was performed to compare the liver toxicity induced in B6C3F1 mice by CD40-targeted Ad-SV40 TAg and untargeted Ad-SV40 T-Ag. Real-time PCR analysis of Ad DNA in the liver samples was used to assess targeting of liver in B6C3F1 mice by the untargeted and CD40-targeted Ad. PET scans were performed to measure the expression of the transgene TK (thymine kinase) in the livers of B6C3F1 mice immunized with CD40-targeted and untargeted Ad expressing TK (*thymidine kinase*).

Results:

1. DCs transduced by using CD40-targeted Ad-SV40 T-Ag showed increased expression of SV40 T-Ag compared to untargeted Ad-SV40 T-Ag.
2. The B6C3F1 mice immunized with CD40-targeted Ad-SV40 T-Ag showed a greater Cytotoxic T Lymphocyte response than mice immunized with untargeted Ad-SV40 T-Ag.
3. There was reduced liver toxicity in B6C3F1 mice that were immunized with CD40-targeted Ad-SV40 T-Ag than the mice immunized with untargeted Ad-SV40 T-Ag as per morphological and histological examination.
4. CD40-targeted Ad vectors showed lower transduction of liver cells as compared to untargeted Ad vectors as shown by real-time PCR analysis of Ad DNA and expression of transgene (TK) in liver samples of both groups.

Conclusion: These results demonstrated that CD40-targeted Ad vaccine is a more potent and a safer vaccine than the untargeted Ad vaccine.